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Genetic Causes of Male Infertility in Gaza Strip- Palestine: A Combined Cytogenetic and Y Chromosome Microdeletions Study

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute, except where due acknowledgment has been made in the text.

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ABSTRACT

Infertility is an extraordinary public health problem, especially in our Arab world, affecting about 15% of couples seeking children. However, in 50% of these the male partner is responsible for infertility. Chromosomal anomalies and Y chromosome classical microdeletions in the azoospermia factor (*AZF*) regions are known to be associated with spermatogenic failure. In addition, partial *AZFc* deletions have been exposed to continuing intense debate on whether they cause spermatogenic failures or not. In the current study, 85 patients with primary male infertility were studied in order to explore the cytogenetic and molecular background of male infertility in Gaza Strip of Palestine. Of the 85 infertile males, 8 patients (9.4%) showed chromosomal anomalies in the form of Klinefelter's syndrome [4/8, 50%], 47,XY,+mar [1/8,12.5%], 46,XY,del(17)(q25) [1/8,12.5%], 45,XY, Robertsonian fusion (15;21) [1/8,12.5%], and one with chromosomal instability that showed multiple mosaic karyotypes (1/8,12.5%). No Y chromosome classical microdeletions could be detected in any of the 85 infertile men, suggesting that ethnic factors, genetic background, and Y chromosome haplogroups are key factors in such deletions. On the other hand, 6 gr/gr *AZFc* partial deletion cases (6/85, 7%) and one b1/b3 *AZFc* partial deletion pattern (1/85, 1.2%) were detected in the infertile group, while one gr/gr deletion was detected in the proven fertile controls (1/30, 3.3%).

In conclusion, our study proves that cytogenetic analysis is mandatory in any diagnostic workup of infertile males. Moreover, our study shows that the incidence of Y chromosome microdeletions is rare in our population, suggesting that other genetic, epigenetic, nutritional and local factors may be responsible for idiopathic azoo/oligozoospermic in Gazian Palestinian population. In addition, we suggest that gr/gr deletions may not be associated with male infertility.

Key words: Male infertility, Chromosomal anomalies, Y-chromosome microdeletions, *AZFc* partial deletion

الأسباب الجينية لعقم الرجال في قطاع غزة-فلسطين:
دراسة مشتركة تتضمن الخلل الكروموسومي والحذف في كروموسوم Y

ملخص الدراسة:

العقم مشكلة صحية عامة غير اعتيادية خاصة في عالمنا العربي اذ تصيب حوالي 15% من الأزواج الراغبين بالإنجاب والرجل يكون مسئولاً عن 50% من هذه الحالات. الاضطرابات و الاعتلالات الكروموسومية والحذف الكلي في مناطق الـ *AZF* من كروموسوم Y من المسببات المعروفة بارتباطها بقصورانتاج الحيوانات المنوية وبالتالي العقم، هذا بالاضافة للجدل القائم حول كون القطع الجزئي لمنطقة *AZFc* في كروموسوم Y مسبباً أيضاً لهذا القصور أم لا. شملت الدراسة 85 مريضاً يعانون من عقم الرجال الأولي بهدف الكشف عن المسببات الكروموسومية والجينية لعقم الرجال في قطاع غزة بفلسطين. حيث أظهرت الدراسة وجود اعتلال كروموسومي لدى ثمانية مرضى ممن شملتهم الدراسة موزعة كالتالي: بصورة متلازمة كلاينفلترز(47,XXY) [4/8 ، 50%]، 47,XY,+mar [1/8، 12.5%] ، 46,XY,del(17) (q25) [1/8، 12.5%] ، 45,XY, Robertsonian fusion (15;21) [1/8، 12.5%]، وحالة واحدة امتازت بتعدد انواع التشريط الكروموسومي وشخصت الحالة على انها تعاني من عدم استقرار الكروموسومات [1/8، 12.5%]. كما أظهرت الدراسة غياب الخلل الناجم عن الحذف الكلي في مناطق الـ *AZF* من كروموسوم Y في عينة الدراسة مفترضاً أن العوامل العرقية والجينية ونوع كروموسوم الذكورة (Y) هم عوامل رئيسة في عمليات الحذف الكروموسومي في كروموسوم Y. من الناحية الأخرى كانت هناك 6 حالات قطع جزئي في منطقة *AZFc* من نوع gr/gr (6/85 ، 7%) وحالة واحدة من نوع b1/b3 (1/85، 1.2%) تم اكتشافها في عينة الدراسة المرضية، بينما كانت هناك حالة واحدة من نوع gr/gr في العينة الضابطة (1/30، 3.3%). أستنتجت الدراسة لزوم وتأكيد إجراء الفحص والتشريط الكروموسومي لتشخيص حالات العقم لدى الرجال في مجتمعنا. كما أظهرت الدراسة ندرة حدوث الخلل الناجم عن الحذف الكلي الواقع في مناطق *AZF* من كروموسوم Y في مجتمعنا مفترضاً وجود عوامل أخرى مسببة لضعف أو إنعدام إنتاج الحيوانات المنوية (اللانطفية) عند الرجال في مجتمعنا الغزّي الفلسطيني كعوامل جينية أخرى وغذائية ومحلية وبيئية، إضافة إلى فرضيتنا بعدم وجود ارتباط بين الحذف الجزئي من نوع gr/gr وقصور انتاج الحيوانات المنوية.

الكلمات المفتاحية: عقم الرجال، الاعتلالات الكروموسومية، الحذف في كروموسوم Y، القطع الجزئي في منطقة *AZFc* من كروموسوم Y.

DEDICATION

This work is dedicated to:

My great parents who have given me faith, love and support through the years

My wife Nisreen and children, Fady, Mennat-Allah, and Mohammed for their enduring patience, love, joy, sustained support and understanding

My brothers and sisters

The people of Palestine who suffered and struggled for free Palestine, this work may contribute for their continuity

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ABBREVIATIONS

A	Adenine
AZF	Azoospermia Factor
bp	base pair
C	Cytosine
CCD	Charged Capture Device
CDY	Chromodomain Y gene
CF	Cystic Fibrosis
DAZ	Deleted in Azoospermia Gene
DBY	DEAD box Y gene
DFFRY	Drosophila Fat Facet related Y gene
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EAA	European Academy of Andrology
EMQN	European Molecular Genetics Quality Network
EtBr	Ethidium Bromide
FBS	Fetal bovine serum
FISH	Fluorescent in-situ hybridization
HS	Hypospermatogenesis
FSH	Follicle Stimulating Hormone
F	Forward
G	Guanine
GTG	G banding using trypsin and Giemsa
hg	Haplogroup
HERV	Human Endogenous Retrovirus
H_2O	Water
HSFY	Heat Shock Transcription Factor Y
ICSI	Intra-Cytoplasmic Sperm Injection
KFS	Klinefelter's Syndrome
Kb	Kilo base pair
LH	Leutinizing hormone
MI	Mitotic Index
M/ml	Million/ml
MSY	Male-specific region of the Y chromosome
Mb	Mega base pair
NAHR	Nonallelic Homologous Recombination
NRY	Non-recombining region of the Y chromosome
OD	Optical density
P	Palindrome
PARS	Pesudo-Autosomal Regions
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
Q banding	Quinacrine banding
R	Reverse
RBCs	Red blood Cells

RBM	RNA-binding motif
RBMY	RNA-binding motif of Y
RNA	Ribonucleic acid
rpm	Revolution per minute
RT	Room temperature
RT-PCR	Real-time PCR
SCO	Sertoli cell-only
SCOS	Sertoli cell-only syndrome
SFV	Sequence family variant
SGA	Spermatogenic arrest
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SPSS	Statistical Package for the Social Sciences
SRY	Sex determination region of Y gene
STS	Sequence tagged site
T	Thiamine
TESE	Testicular sperm extraction
TTY	Testis Specific Transcription Y
TAE	Tris Acetate EDTA buffer
WHO	World Health Organization
UV	Ultra violet
UEP	Unique event polymorphism
USP9Y	Ubiquitin specific protease 9, Y chromosome
UTY	Ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome
YCC	Y chromosome Consortium
Yp	Y chromosome short arm
Yq	Y chromosome long arm
ZFY	Zinc-Finger Y

Chapter One

INTRODUCTION

1.1. Overview

Infertility is a real extraordinary problem worldwide, especially in Arab cultures, since it exhausts the couples psychologically, socially, and emotionally; furthermore, it drains their financial resources. Infertility has been defined by the world health organization (WHO) as the inability to conceive naturally after at least one year of unprotected intercourse (Dohle *et al*., 2004). It is estimated that as many as 15% of couples worldwide who seeks children suffer from infertility (Pryor *et al*., 1997; Tse *et al*., 2000; Dada *et al*. ,2003; Hellani *et al*., 2006).

Male causes for infertility are found in about 50% of infertile couples (Pryor *et al*., 1997; Ambasudhan *et al*., 2003). Reduced male fertility can be a result of congenital and/or acquired abnormalities. They include infections of the genital tract, varicocele, developmental and anatomical abnormalities, endocrinopathies, immunological factors, environmental exposures, and genetic abnormalities. Frequently, however, male infertility is difficult to diagnose, and about 60-75% of cases remain idiopathic. These idiopathic cases present with no previous history associated with fertility problems and have normal findings on physical examination (Dohle *et al*., 2004).

Contemporarily, a considerable and substantial attention has been focused on the role of genetic factors in spermatogenesis failure. It has been estimated that over 4000 genes are involved in the genetic control of human spermatogenesis (Gianotten *et al*., 2004). Some of them have been outlined and a number of genes related to infertility have been cloned, while other genes have been assigned to specific chromosome regions, but the majority of them remain undeciphered. Genetic anomalies causing male infertility can be roughly classified into three major groups: (1) chromosomal aneuploidies and rearrangements where batteries of genes on specific chromosomes have increased/decreased their expression dosage or changed their normal genomic environment; (2) interstitial deletions, whether microscopic or

submicroscopic, where deletions or rearrangements of multiple genes mapped in a molecular neighborhood have changed their normal expression pattern, and (3) single gene defects where the expression of a single key element gene is changed or lost causing then male infertility (Vogt, 2004).

Aneuploidies can be easily diagnosed after performing G banding using trypsin and Giemsa (GTG) karyotyping. Therefore, GTG karyotyping is certainly a mandatory test in the diagnostic workup of any infertile man.

Interstitial deletions of the Y chromosome are the most common structural abnormalities. The majorities of these deletions are submicroscopic and can escape detection by GTG karyotyping. Therefore, only molecular techniques, such as polymerase chain reaction (PCR) can detect them.

The role of Y chromosome in male infertility was first elucidated in 1976 when Tiepolo and Zuffardi have proposed the existence of a key factor which controls spermatogenesis encoded by a gene that is localized within the euchromatic region of the Y chromosome long arm (Yq11), which was called the azoospermia factor (*AZF*), because the first six men observed with microscopic terminal deletions in Yq, by routine karyotyping, were azoospermic (Tiepolo and Zuffardi, 1976). This substantially proved the close association between Y chromosome deletions and male infertility. In 1996, Vogt together with his colleagues have observed that Y chromosome microdeletions follow a certain deletion pattern, with three distinct recurrently deleted non-overlapping subregions in proximal, middle, and distal Yq11, designated "*AZF*a," "*AZF*b," and "*AZF*c," respectively. Many genes controlling spermatogenesis where mapped within these *AZF* regions (Foresta *et al*., 2001).

Interstitial and terminal deletions in *AZF*a, or *AZF*b, or *AZF*c alone or in any combination of the Y chromosome long arm (Yq) are all associated with dramatic nonobstructive spermatogenic failure. Therefore, there is a clear cause-effect relationship between *AZF* loci deletion/s and male infertility (Reijo *et al*. 1996a; Simoni *et al*., 1998; Vogt, 1998; Foresta *et al*., 2001; Repping *et al*., 2002; Krausz *et al*., 2003; Krausz *et al*., 2006a; Fernando *et al*., 2006). These gross "microdeletions" are associated with divergent testicular histological profiles, ranging from Sertoli cell-only syndrome (SCOS), hypospermatogenesis (HS) to spermatogenic arrest (SGA) (Vogt *et al*., 1996).

Apart from infertility, men presenting with *AZF* microdeletions appear otherwise healthy. These microdeletions are usually detected by performing sequence tagged site (STS) based PCR techniques on patient peripheral blood genomic DNA (Y chromosome microdeletion assay by PCR). Therefore, in addition to GTG karyotyping, Y chromosome deletion detection by PCR is also a mandatory test in the azoo/oligozoospermic patient workup. Worldwide, Y chromosome microdeletion assay has become a routine test (Ferlin *et al*., 2007). The literature estimate places about 10% cases of idiopathic azoo/oligozoospermia due to deletion in *AZF* regions, therefore, it is acknowledged as the most common molecularly diagnosable cause of spermatogenic failure in men with nonobstructive azoospermia or severe oligozoospermia (Reijo *et al*., 1995; Saxena *et al*., 2000; Kuroda-Kawaguchi *et al*., 2001; Foresta *et al*., 2001; Repping *et al*., 2003; Machev *et al*., 2004; Aknin-Seifer *et al*., 2004; Foresta *et al*., 2005). The literature also showed that complete deletion of the *AZF*c region which spans 3.5 Mb of the Y chromosome is the most common known genetic cause of human male infertility (Saxena *et al*., 2000; Kuroda-Kawaguchi *et al*., 2001; Fernandes *et al*., 2002; Repping *et al*., 2003; Machev *et al*., 2004; Nathanson *et al.,* 2005; Fernando *et al*., 2006; Ferlin *et al*., 2007).

Yq11 human chromosome deletions most frequently occur as *de novo* mutation events in men with idiopathic azoospermia or oligozoospermia, therefore these deletions are not detected in their fathers or passed to their siblings. Some patients with these deletions can father, but usually not naturally, rather by assisted reproductive techniques, e.g., by testicular sperm extraction (TESE) in conjunction with intracytoplasmic sperm injection (ICSI) techniques.

Y chromosome microdeletion screening provides diagnostic, prognostic, and preventive impacts. In addition, it can improve genetic counseling. Moreover, infertile men are known to be at increased risk of androgen deficiency and testicular neoplasia (Giannouli *et al*., 2004; Nathanson *et al*., 2005); however, whether the patients with Yq deletions have a greater risk also requires careful monitoring.

Apart from the aforementioned complete *AZF* region deletions (also called classical deletions), a new type of Yq deletion has recently attracted the

attention. Partial deletion patterns within the *AZFc* region have been proposed and then described. The spermatogenic effect of these deletions was found to vary among different populations.

Hitherto, no one has studied or characterized the genetic causes of male infertility in Gaza Strip (Palestine). To do so we have employed GTG karyotyping and PCR STS based techniques to detect the aforementioned genetic causes in the azoo/oligozoospermic Gazian patients.

1.2. Aim of the study

The overall objective of the present study was to determine the prevalence of numerical and structural chromosomal abnormalities among idiopathic azoospermic and oligozoospermic infertile males in Gaza Strip.

1.3. Objectives of the study

- Establish the prevalence of numerical and structural chromosomal abnormalities that can be detected by GTG karyotyping in Gaza Strip azoospermic and oligozoospermic infertile men.
- Get hands on experience in performing and interpreting GTG karyotyping.
- Establish the prevalence and molecular characteristics of Y chromosome microdeletions in *AZF* subregions in Gaza Strip azoospermic and oligozoospermic infertile men.
- Assess for the first time the occurrence of partial *AZFc* deletions in Palestinian men.
- Establish the association between partial *AZF*c microdeletions and spermatogenesis failure.
- Establish guidelines on male infertility workup in Gaza Strip and Palestine.

Chapter Two

REVIEW OF LITERATURE

2.1. Infertility

Children are the usual and expected outcome of any legal and stable marriage relation. The inability to conceive naturally after one year of regular unprotected intercourse between married couples defines infertility by the WHO. It is estimated that approximately 15% of married couples are infertile according to the aforementioned definition (Pryor *et al*., 1997; Tse *et al*., 2000; Dada *et al*., 2003; Hellani *et al*., 2006). Infertility affects both men and women. Male factor either is a sole cause or contribute to the infertility problem in about 50% of the involuntarily childless couples (Pryor *et al.*, 1997; Ambasudhan *et al*., 2003).

2.2. Etiology of male infertility

Male infertility is manifested as quantitative abnormality (azoospermia, cryptozoospermia and oligozoospermia), or as qualitative abnormality (asthenospermia, teratozoospermia and necrospermia) or both (Hargreave, 2000a; Ferlin *et al*., 2007). The causes of male infertility are numerous and can be divided into five major diagnostic categories: (i) disorders related to motility or sperm function; (ii) disorders related to obstructive lesions; (iii) disorders related to spermatogenic failure; (iv) sexual dysfunction disorders of erection and ejaculation; (v) endocrine dysfunction. Both environmental and genetic factors, combined or separate, have been suggested (Vogt, 2005a). No clear causal factor could be diagnosed in about 50% of cases coining the term "idiopathic male infertility". These men usually present with no previous history associated with fertility problems and have normal findings on physical examination. Genetic abnormalities contribute to a fair enough percentage of these idiopathic cases (Vogt, 2004).

Assessing male fertility potential starts with a thorough medical and reproductive history, a physical examination and a semen analysis. Whether the male partner is presenting with primary infertility (has never initiated a pregnancy in the past), most of the idiopathic male infertility cases are

categorized to this type, or secondary infertility (has fathered a child with the same or a different partner in the past) may help to detect the specific cause of infertility. About 67-71% and 29-33% of patients have primary and secondary infertility, respectively (Irvine, 1998; Seshagir, 2001).

A past medical history can reveal information that is very helpful. Sexually transmitted diseases can result in obstruction of the tubular path. The childhood illness mumps may affect the testicles if contracted after puberty, causing mumps orchitis, which can result in severe inflammation of one or both testes with subsequent scarring, and reduces the ability of the testes to produce sperm.

Previous abdominal or scrotal surgical procedures, such as hernia repair or hydrocelectomy, can potentially block the transport of sperm. Traumatic injuries to the pelvis and testicles may also impair normal spermatogenesis or transport. Illnesses such as diabetes mellitus (DM) or respiratory tract disease, such as cystic fibrosis (CF), are in some cases associated with abnormalities of sperm transport as well.

Several environmental factors and agents such as heat, radiation, heavy metals, organic solvents and pesticides have been shown to affect spermatogenesis as have medications such as Azulfidine® (used for ulcerative colitis) and Tagament® (used for gastrointestinal ulcers), and the drugs caffeine, nicotine, alcohol, marijuana and cocaine. The antibiotic nitrofurantoin and others have been shown to inhibit sperm formation.

A thorough physical examination should be done, the testicles are assessed for normal size and consistency. Small, soft testes suggest impaired sperm production. The presence and consistency of the epididymis, and the presence of the vas deferens is confirmed. The vasa deferentia is also examined; as some men may have an absence, either partial or complete, of one or both vasa deferentia. The scrotum is examined for a varicocele, the most common cause of infertility in men (Pryor *et al*., 1997; Dohle *et al*., 2004). If present, a varicocele, which is an enlarged vein, allows blood to collect near the testicle, and consequently an abnormal amount of heat is transmitted to the testicle which may affect sperm production and production of the male hormone testosterone. A rectal examination is done to evaluate the prostate gland, which contributes fluid to the semen volume.

2.2.1. Idiopathic male infertility

Idiopathic male infertility is often associated with genetic and epigenetic abnormalities. The possibility that many cases of idiopathic male infertility might have genetic background was bolstered by the failure of most clinical therapies to correct deficient spermatogenesis (Silber *et al*., 1995b; Devroey *et al*., 1998). Genetic causes account for 10-15% of severe male infertility cases (Reijo *et al.*, 1995; Vogt *et al*., 1996; Ferlin *et al*., 2006). Such abnormalities include chromosome translocations and aneuploidies, Y chromosome microdeletions, and androgenic receptor gene mutations. Numerous other genes are likely to be associated with male infertility. Initial reports from human studies have identified several candidate genes, including *Protamine-1* and *Protamine-2* genes, *DAZ*L1, *SPO11*, *EIF5A2*, *USP26*, and others. Thus, genetic causes are either detected at the cytogenetic and/or at the molecular levels. In addition to gene mutations and polymorphisms, damage to the chromatin resulting in single and double strand DNA breaks affects male fertility. Epigenetic abnormalities such as gene imprinting also may contribute to male infertility.

2.3. Chromosomal aberrations causing male infertility

Chromosomal aberrations whether numerical or structural are found in about 14% of azoospermic men and 5% of oligozoospermic men (Johnson, 1998; Olesen *et al*., 2001; Foresta *et al*., 2002a; Vogt, 2004; Foresta *et al*., 2005), these aberrations play a prime role in male infertility with abnormal semen parameters (Nagvenkar *et al*., 2005). Chromosomal aneuploidies and microscopic structural abnormalities are usually detected by cytogenetic methods, e.g., GTG karyotyping and Fluorescence Insitu Hybridization (FISH) techniques, but molecular techniques also can detect them such as Y chromosome microdeletions.

Most of the aneuploidies causing male infertility include one of the sex chromosomes, X and Y, whereby the karyotype 47,XXY which is associated with the Klinefelter syndrome (KFS) constitute the largest group. About 15% of KFS are classified as mosaic, with a 46,XY/47,XXY karyotype. Patients with KFS are characterized by testicular hypotrophy, azoospermia, elevated plasma FSH levels, and subnormal intelligence. However, severe

oligozoospermia occasionally may be present in patients with KFS, especially in those with the mosaic karyotype (Ferlin *et al*., 2006). There are few reports in which KFS patients have fathered biologically, but only through TESE and ICSI assisted reproductive techniques. (Tournaye *et al*.,1996)

Klinefelter patients with increasing number of X chromosomes, (48, XXXY) and (49,XXXXY), are KFS variants which shift their sexual phenotype to the female side. This suggests an X-chromosome dosage effect on the males gonad development, and also shows that the balance between X and Y chromosomes is crucial (Vogt, 2004).

Double Y chromosome men (47,XYY) are less frequent than the KFS with its variants, but with a variable infertility status, and if they are fertile their phenotype is usually oligozoospermia (Vogt, 2004). Characteristically these patients are aggressive in nature.

The mosaic karyotype 45,X0/46,XY leads to male infertility because of mixed gonad dysgenesis. It is found in infertile men with an incidence of 4%. Noteworthy, azoospermia and the karyotype 45,X0 (Turner syndrome) can be seen in men with *AZF*c deletions, this is because of terminal/distal deletion of the Yq11, rendering the Y chromosome unstable. Therefore, these patients belong to the *AZF* patient group.

Azoospermic men with the karyotype 46,XX (46,XX maleness, *SRY*-positive XX male) are scarce in frequency and they are invariably sterile. *AZF*a, b, and c regions are all absent (*AZF*a-b-c deletion) in these men. These patients have fully mature male genitalia, normal height and unimpaired intelligenece (Ferlin *et al*., 2006). Intrestingly, Rajender *et al*. (2006) reported a case of *SRY*-negative 46,XX male with mature male genetalia but infertility. This case was also characterized by *AZF*a-b-c deletion.

Autosomal chromosomal anomalies, either aneuploidies, balanced Robertsonian translocations, balanced reciprocal translocations, balanced inversions, deletions, or extra markers were found less frequently than sex chromosome abnormalities (Vogt, 2004; Ferlin *et al*., 2006). Structural aberrations are found 10 times more frequently in infertile men than in the fertile men population (Vogt, 2004).

The most frequent structural chromosomal abnormalities in human is Robertsonian translocations and can affect male fertility. Robertsonian

translocations occur only between two acrocentric chromosomes (i.e., chromosomes #13, 14, 15, 21, and 22) which result in a single abnormal chromosome, generally, dicentric and contains most of the long arms of the original two chromosomes and subsequent loss of there short arms. The resulting balanced karyotype has only 45 chromosomes including the translocated one. In 60% of cases with Robertsonian translocations the two acrocentric chromosomes, 13 and 14, were involved (Vogt, 2004), the second most common is between chromosomes 14 and 21 (Ferlin *et al*., 2006).

Pericentric inversions in chromosomes 1, 3, 5, 6, and 10 are associated with reduced rate of sperm production and therefore may lead to male infertility. The expected phenotype ranges from oligozoospermia to azoospermia.

Male infertility caused by (Y;autosome) translocations have variable impact, since same karyotype was observed in fertile as well as in infertile men sometimes even within the same pedigree (Vogt, 2004). In most (Y;autosome) cases, the distal heterochromatin block of the Y long arm is translocated to the short arm of an acrocentric chromosome.

2.4. The role of human Y-chromosome in male infertility

The Y chromosome is not essential for life, but also it is not a genetic wasteland. Its main task is to ensure that men can make and deliver sperm for the continuity of human species. In addition, the male sex determination role of the Y chromosome has been known for a long time, since it carries the sex determining region Y (*SRY*) gene, which switches the development of the indifferent gonad from the default female pathway to the male pathway, which results in the development of the testis (Goodfellow and Lovell-Badge, 1993; Yen, 2001). It harbors fewer genes through its 60 Mb length in comparison to the other 23 chromosomes, even when compared to its counterpart, the X chromosome (Carvalho and Santos, 2005).

In the human Y-chromosome, meiotic crossovers and recombination with the X chromosome take place only in its two relatively short regions, the Yp-PAR1, and the Y-q-PAR2 (PAR= Pseudoautosomal region), found at the tips of Yp and Yq, respectively. The rest of the Y chromosome regions which compromise 95% of the entire Y chromosome are called NRY (Non-

recombining region of the Y chromosome), which are flanked by the PAR1 and PAR2. Skaletsky *et al*. (2003) renamed NRY to MSY (male-specific region of the Y chromosome) because they observed that the designation NRY did not reflect the dynamic evolutionary events occurring in this part of the Y chromosome.

The Y-chromosome is paternally inherited and forms 2-3% of the haploid genome, most of which is heterochromatic. The euchromatic portion of MSY only encompasses 23 Mb that contains 156 transcription units including 78 protein-coding genes that collectively encode 27 distinct proteins (Skaletsky *et al*., 2003; Simoni *et al*., 2004). The MSY is made up of three main gene groups. The first group is composed of X-transposed genes, represented by two coding units approximately 99% identity to the corresponding X chromosome. The second group consists of 16 coding genes that are generally single-copy and expressed ubiquitously. These genes are involved in housekeeping cellular activities, and are probably involved in functions other than male reproduction. The third group is formed of ampliconic multicopy class genes that encompasses approximately 10.2 Mb distributed in Yp and Yq, which consists of large regions where gene copies show near absolute sequence identity (~99.9%), they are exclusively testis specific, and are implicated mainly in spermatogenesis and male fertility (Skaletsky *et al*., 2003).

2.4.1. Y- chromosome haplogroups and sperm count

Many Y-chromosome polymorphisms on the MSY, also called single nucleotide polymorphisms (SNPs) or unique event polymorphisms (UEPs), are biallelic markers, and can be combined in haplogroups that define Y chromosome lineage with specific geographic distributions around the world (Carvalho and Santos, 2005; Ferlin *et al*., 2006). These haplogroups are defined by the SNPs/UEPs, until now, more than 200 Y specific SNPs have been identified (Krausz and Degl'Innocenti, 2006b). Few published studies showed correlation between specific Y-chromosome haplogroups and low sperm counts. Krausz *et al*. (2001) have found that one class of Y chromosome referred to as haplogroup 26+ (hg 26+; or hg K according to YCC, 2002 nomenclature) was associated with unexplained reduced sperm

counts in individuals from the Danish population. Moreover, Kuroki *et al*. (1999) have also reported a haplogroup termed by the authors as Y haplogroup II (D2b according to YCC, 2002 nomenclature) to be more common in azoospermic Japanese men when compared with normal Japanese men. On the contrary, Carvalho and Santos (2005) did not detect any statistical association between Y-chromosome haplogroups and male infertility. In addition, Paracchini and co-workers (2000) failed to find any correlation between Y haplogroups and different sperm counts in Italian population.

2.4.2. Y-chromosome: The interval deletion map

The Y chromosome has been divided by Vergnaud *et al*. (1986) into 7 deletion intervals (1 to 7) (**Fig.2.1**): the short arm and the centromere contain intervals 1-4, distal to proximal; the euchromatic part of Yq is represented by intervals 5 and 6, proximal to distal; the heterochromatic region is defined as interval 7. In 1992, Vollarth and colleagues further divided the seven intervals map into 43 subintervals, and this is the most commonly used map (**Fig. 2.1**) (Foresta *et al*., 2001; Dada *et al*., 2003). The 43 interval deletion map of human Y chromosome is based solely on PCR technology which contained an ordered array of DNA landmarks or specific monomorphic molecular markers that occurs only once in the human genome, called STSs (sequence tagged sites), across the entirety of the Y chromosome. These detailed molecular deletion mapping studies have explored many of the Y chromosome defects, especially deletions and microdeletions, in addition, it revealed there exact breakpoints (Vogt, 2005b).

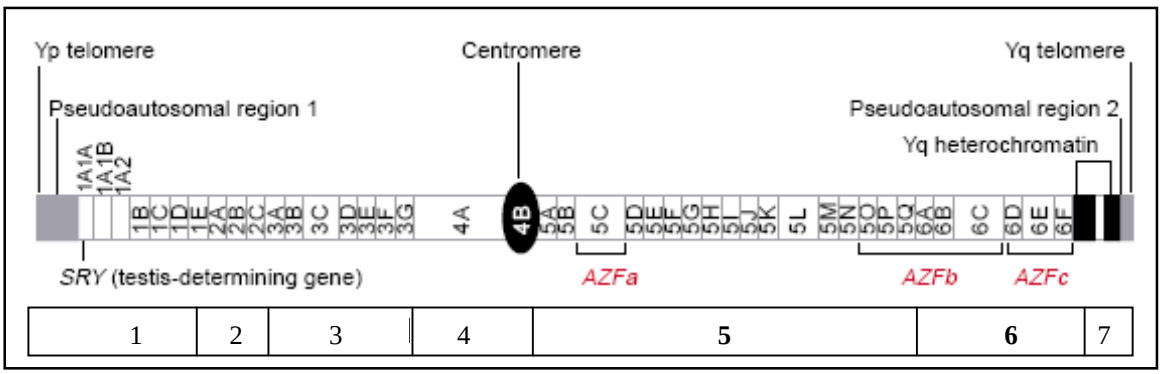

Figure 2.1. Y-chromosome: schematic presentation of the intervals that have been defined by deletion mapping and PAR 1 and 2 (adapted from Nabeel A. Affara, 2003).

2.4.3. Y-chromosome long arm deletions and male infertility

Tiepolo and Zuffardi (1976) first observed human Y chromosome deletions that interfere with spermatogenesis causing male infertility and sterility 30 years ago (Tiepolo and Zuffardi,1976). After the cytogenetic analysis of six azoospermic patients by Q banding (Quinacrine banding), terminal deletions of the long Y chromosome arm (Yq) were visible. This indicated that their Y deletions included the entire Yq12 heterochromatin block and an undefined amount of the adjacent active euchromatic part of Yq (Yq11.23). Furthermore, they found the fathers of two of their Y deleted patients carry a normal Y chromosome indicating that these deletions were *de novo* events. Consequently, the authors postulated that a key genetic Y factor critical for spermatogenesis is located within the distal Yq11, and its deletion was the cause of the azoospermia of their six patients. This factor was termed as the azoospermia factor (*AZF*). This also was then confirmed by additional numerous studies at both the cytogenetic and molecular levels (Vogt *et al.,* 1992; Ma *et al*., 1992; Ma *et al*., 1993; Nagafuchi *et al*., 1993). The results of these studies supported the concept that numerous Y chromosome genes impinge on spermatogenic maturation process of the male germ cell. In 1996, Vogt and co-workers screened 370 men with idiopathic azoospermia or oligozoospermia for deletions, using 76 STSs, and their findings suggested the existence of three nonoverlapping regions within the *AZF* locus; designated from proximal to distal, as "*AZF*a", "*AZF*b", and "*AZF*c", respectively. These regions have been mapped within the deletion intervals 5 and 6 of the Y chromosome, which lie within Yq11.21-Yq11.23.

At least three different intervals (i.e., the *AZF*a, *AZF*b, and *AZF*c) on the Y chromosome long arm (Yq11) are critical for male germ cell differentiation. They encode specific genes essential for the normal complex process of spermatogenesis. These intervals vary in length as they also vary in the encoded genes. Most of the *AZF* encoded genes are testis specific. The exact role of the candidate *AZF* genes is largely yet unknown due to both the extreme rarity of naturally occurring *AZF* gene specific mutations and the lack of functional assays (Krausz *et al*., 2006a). Structural abnormalities in these intervals, notably microdeletion events, were found to be the most prominent

in azoospermic and oligozoospermic men with a normal karyotype (46,XY) (Reijo *et al*., 1995,1996a). These microdeletions are only detectable by molecular techniques, because the deletion length is less than to be visualized or discerned by routine cytogenetic methods, and this is why they are called microdeletions. Patients can show different deletion patterns, one or more *AZF* loci could be deleted (e.g. *AZF*b and *AZF*c), or even portions of variable lengths of one *AZF* locus may be deleted (partial microdeletions, e.g., gr/gr microdeletion, discussed below). Large scale of studies showed that the most frequently deleted region is *AZF*c, followed by deletions of the *AZF*b and *AZF* b-c or *AZF* a-b-c regions whereas deletions of the *AZF*a region are extremely rare (Saxena *et al*., 2000; Martinez *et al*., 2000; Tse *et al*., 2000; Foresta *et al*., 2001, Akbari Asbagh *et al*., 2003; Krausz *et al*., 2003; Ambasudhan *et al*., 2003; Yu *et al*., 2004; Carvalho and Santos, 2005; Fernandes *et al*., 2006; Omrani *et al*., 2006). Molecular studies have demonstrated that microdeletions at Yq11 (*AZF* loci) may represent the etiological factor in 10-15% of cases with idiopathic azoospermia or severe oligozoospermia (Reijo *et al*, 1995; Vogt *et al*., 1996; Ferlin *et al*., 2003). No clear correlation exists between the size and localization of the deletions within the *AZF* loci and the testicular phenotype. However, it is obvious that more widened deletions, as more genes are deleted, are associated with the most severe spermatogenic failure (Foresta *et al*., 2001). Most if not all of the deletions in the *AZF* loci are *de novo* in origin, i.e., they are not polymorphic events, which strengthen their pathogenic role in determining the spermatogenic disruption (Foresta *et al*., 2000). The transmission of these deletions to male offspring is obligatory, whether transmitted naturally or more often by assisted reproduction techniques (Oates *et al*., 2002; Hellani *et al*., 2006). Quite surprisingly, Oates *et al*. (2002) reported a case of fraternal twins with *AZF*c deletion naturally conceived from an intact-*AZF*c father. On the other hand, there are few exceptional case reports in the literature that showed that *AZF* loci deletions can be naturally transmitted to the male offspring (Stuppia *et al*, 1996; Pryor *et al*., 1997; Chang *et al*., 1999; Kamischke, 1999; Saut *et al*., 2000; Rolf *et al*., 2002; Calogero *et al*., 2002; Gatta *et al*., 2002; Kuhnert *et al*., 2004). These reports are contradicted only in one aspect, some of them showed that the deletions were identical with no

change or amplification between the father and his son(s) (e.g. Pryor *et al*., 1997; Saut *et al*., 2000; Gatta *et al*., 2002; Kuhnert *et al.*, 2004); whereas others showed that the deletions where more widened and amplified in the male offspring (e.g. Stuppia *et al*, 1996; Calogero *et al*., 2002). In this context, it is worth to note that few people who harbor these microdeletions can father naturally, which obviously shows the heterogeneity in phenotypes associated with the different microdeletion patterns, as it also exemplify the concept that fertility is not synonym of normal sperm count (Ferlin *et al*., 2007). Moreover, these previous reports demonstrated that the phenotypes in the sons were deleterious, since all of them were azoospermic, and no one could father naturally, i.e., only by assisted reproduction methods.

DNA sequencing of the Y chromosome has identified unique structural features, eight massive palindromes (mirror image sequences) within Yq being the most pronounced, designated 1 to 8, from distal to proximal, respectively (**Fig.2.2.**). Palindromes consisting of very long, near identical direct and indirect repeats, dubbed amplicons (Skaletsky *et al*., 2003). Most of the multicopy genes are contained within these palindromes. Recombination events between these amplicons is believed to be the cause of the high rate of the *de novo* Y long arm microdeletions that lead to infertility, for instance Kuroda-Kawaguchi *et al*. (2001) showed that 47 out of 48 men with *AZF*c deletion had the same proximal and distal breakpoints in 229 Kb amplicons flanking the *AZF*c region. This was strengthened and supported by the study of Repping *et al*. (2002) who showed that recombination between Yq repetitive palindromic regions can explain the majority of *AZF*b and *AZF*bc deletions. Then it was shown that intrachromosomal nonallelic homologous recombination (NAHR) events are recurrent in the MSY region, mostly at the *AZF*c locus, which explains the high incidence of *AZF*c locus deletion among other *AZF* loci (Fig.2.2) (Skaletsky *et al.*, 2003; Repping *et al.*, 2003; Machev *et al*., 2004; Vogt, 2005b).

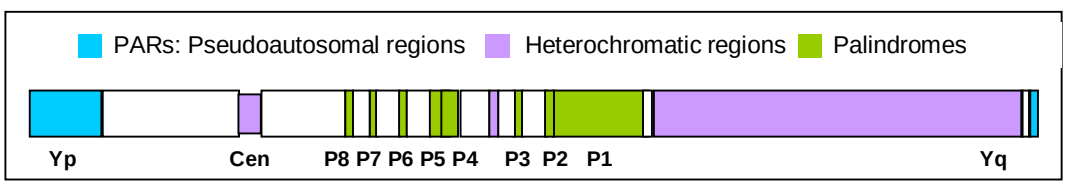

Figure 2.2. The Yq massive palindromes map.

2.4.3.1. *AZF* loci deletions causing male infertility

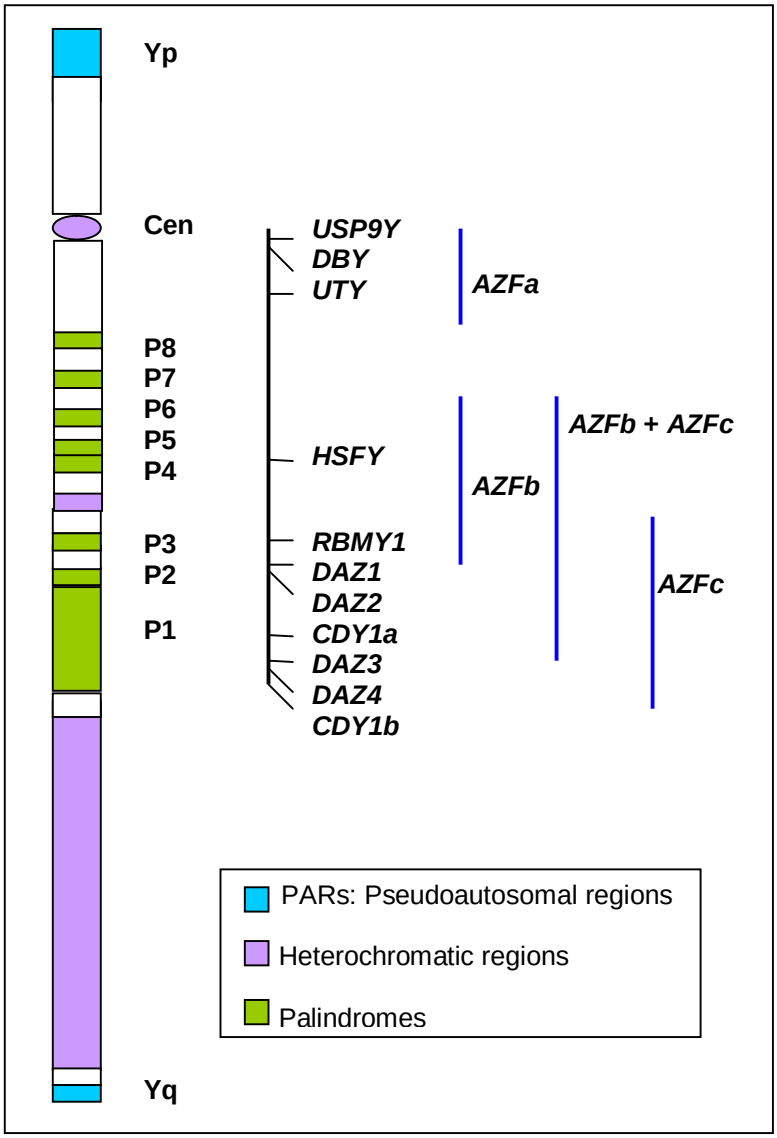

Fig. 2.3. Schematic display of the human Y-chromosome. Showing pseudoautosomal regions (PARS), the heterochromatic regions, the main genes on the euchromatic region, and the *AZF* regions associated with male infertility (adapted from Skaletsky *et al*., 2003, and Jobling and Tyler-Smith, 2003).

2.4.3.1.1. The *AZFa* region

AZFa locus approximately spans 800 kb (Sun *et al*., 2000; Ferlin *et al*., 2003). Three single copy genes were precisely mapped in the *AZFa* interval (deletion interval 5 C/D) named from proximal to distal Yq ubiquitin-specific protease 9, Y chromosome gene (*USP9Y*), DEAD box Y gene (*DBY*), and ubiquitously transcribed tetratricopeptide repeat Y chromosome gene (*UTY*)

(Sun *et al*., 1999; Foresta *et al*., 2000). The specific role of the three genes in human spermatogenesis is still to be elucidated, and it is debatable which of them is responsible for the *AZFa* phenotype. Interestingly, all of the three *AZFa* genes have an X chromosomal counterpart on the short arm (Xp11.4) which escape X inactivation (Vogt, 2004; Ditton *et al*., 2004; Singh *et al*., 2005; Krausz and Degl'Innocenti, 2006b).

Both *USP9Y* and *DBY* deletions may cause testiculopathies, but Foresta *et al*. (2000) demonstrated that *DBY* gene is the fundamental major *AZFa* candidate because it is more frequently deleted than the *USP9Y* (i.e., partial *AZFa* deletion) and its absence produces a significant reduction of germ cells (marked hypospermatogenesis) or even their complete absence [sertoli-cell-only (SCO)]. This has been also supported by Foresta *et al*. (2001) who stated that *USP9Y* is expressed widely in different tissues including the testes, while *DBY* have a testis specific transcript in addition to ubiquitous transcripts. Vogt (2005b) also suggested that the *DBY* gene is the major *AZFa* gene. This also could be further supported by Krausz *et al*. (2006a) who reported two different cases with two different *de novo* point mutations in the *USP9Y* gene causing an exon to be skipped and protein truncation, both cases are characterized by very mild testicular phenotype. On the light shed by this finding, they predicted that *USP9Y* is more likely to be a fine tuner that improves efficiency rather than a provider of an essential spermatogenic function. Moreover, heretofore, no reports showed an isolated deletion in the *UTY* gene (Foresta *et al*., 2000). Consequently, we can conclude that the *DBY* gene is the actual key player gene within the *AZFa* region, and its deletion is responsible for the *AZFa* phenotype in the *AZFa* region (Foresta *et al*., 2000; Ditton *et al*., 2004). Deletion of both the *DBY* and *USP9Y* genes may exacerbate the patient infertility status (Singh *et al*., 2005).

Deletions of the *AZFa* region are the least common class of *AZF* deletions, and are most frequently associated with azoospermic patients (Sun *et al*., 2000). Low frequency of *AZFa* deletions in comparison to the other *AZF* loci can be attributed to the more conventional and non-repetitive structure of the region. Although, intrachromosomal recombination events between two repetitive specific Human Endogenous Retroviral #15 (HERV15) sequence blocks flanking the *AZF*a region, HERV15q1 and HERV15q2, each ~10 Kb

long, are now believed to be the major cause of *AZFa* deletions (Fig.2.4) (Sun *et al*., 2000; Kamp *et al.*, 2000; Blanco *et al*., 2000; Krausz and Degl'Innocenti, 2006b).

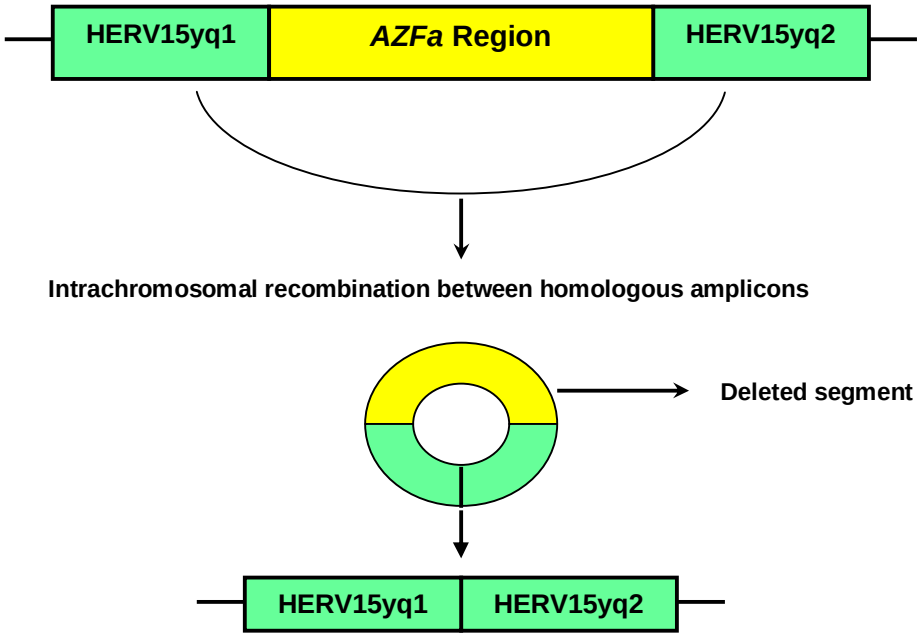

Figure 2.4. Schematic representation of the deletion mechanism in *AZFa* region.

Patients with *AZFa* deletion usually present with more severe phenotype than the most commonly encountered *AZFc* microdeletions. Complete deletion of the *AZFa* region is usually associated with complete Sertoli-cell-only syndrome (SCO) in testicular biopsies (Vogt *et al*., 1996; Foresta *et al*., 2001; Carvalho and Santos, 2005; Vogt, 2005b; Ferlin *et al*., 2006). Foresta *et al*. (2000) showed in their study that the incidence of *AZFa* deletions might be underestimated because they could detect *AZFa* partial deletions (isolated *AZFa* gene deletions) in infertile men in whom Y microdeletion test originally did not show deletion in any of the *AZF* loci. However, when they used the *DBY* specific primer pair they detected six more cases that originally escaped detection.

2.4.3.1.2. The *AZFb* region

AZFb region spans approximately 6.2 Mb and usually mapped to the deletion subintervals 5O-6B, but this distance varies between individuals and according to the screening methodology (Foresta *et al*., 2001; Ferlin *et al*., 2003; Vogt, 2004). The genes that have been mapped to the *AZF*b region include *RBMY1*, *SMCY, eIF-1Y, PRY, RPS4Y2, HSFY, CDY2, TTY2, TTY5, TTY6, TTY9, TTY10, TTY12, TTY13, TTY14, TTY16, XKRY, CYorf14, CYorf15A* and *CYorf15B* genes. Testes specific Transcript Y (*TTY* genes) are described as non-coding genes because they do not have an open reading frame (Lahn and Page, 1997; Skaletsky *et al*., 2003); while the role in spermatogenesis for most of the other genes is still to be elucidated. Originally, *AZFb* was considered a distinct Yq deletion interval (Vogt *et al*., 1996); however, this was disproved by Repping *et al*. (2002) who showed that the previously considered non-overlapping *AZFb* region actually overlaps with the proximal part of the *AZFc* region by 1.5 Mb. The *AZFb-AZFc* overlapping interval includes seven extra genes, *BPY2.1*, *CDY1a*, *DAZ1* (Deleted in Azoospermia), *DAZ2*, *TTY3*, *TTY4*, and *TTY17*, but these genes also have more copies distal to the *AZF*b, i.e., in the unique *AZFc* deletion interval (Vogt, 2004). Repping *et al*. (2002) also demonstrated that homologous recombination between repetitive palindromic regions in Yq can explain most *AZF*b isolated deletions.

Several studies have proposed that the testis specific RNA Binding Motif-Y chromosome (*RBMY1*) gene family that encodes a nuclear protein represents the *AZFb* candidate (Ma *et al*., 1993; Chai *et al*., 1997; Mahadevaiah *et al*., 1998; Friel *et al*., 2002; Carvalho and Santos, 2005). This gene is highly expressed in mitotically active cells, thus may promote mitotic divisions in spermatogonia. Although there are numerous copies and pseudogenes (more than 30 copies) of *RBMY1* on the Y, most of which are nonfunctional (Krausz and Degl'Innocenti, 2006b). However, there appears to be a functional copy in the region just proximal to *AZFb/c* overlapping region, with no rescue homolog elsewhere (Silber *et al*., 1998). On the other hand, the study of Ferlin *et al*. (2003) identified four infertile men with similar *AZFb* deletion breakpoints and all of them still retained the intact *RBMY1* functional

copy (i.e., partial *AZFb* deletion proximal to *RBMY1* gene) and all of them are represented with a testicular histology picture of either SCOS or marked hypospermatogenesis. This assumes that in addition to the *RBMY1* gene there is/are other candidate gene/s within the *AZFb* region that is/are important in the core of the spermatogenesis process. Different studies have unveiled the assumed candidate gene to be the heat-shock transcription factor Y linked (*HSFY*) gene that is mapped in the palindromic sequence P4 in proximal *AZFb*; this gene encodes a novel heat shock protein. *HSFY* is a multicopy (2 copies), and is expressed exclusively in the testis, furthermore, its expression is restricted to both sertoli cells and spermatogenic cells. However, these studies did not define the exact deletion breakpoints. This gene was also found to be deleted in idiopathic azoospermic males (Shinka *et al*., 2004; Tessari *et al*., 2004; Carvalho and Santos, 2005; Vinci *et al*., 2005; Krausz and Degl'Innocenti, 2006b).

Complete *AZFb* deletions (also called classical *AZFb* deletions) are associated with recombination between palindromic sequences and removes 6.23 Mb, which as mentioned earlier extend 1.5 Mb into the proximal portion of *AZFc* (Repping *et al*., 2002). Complete deletion of the *AZFb* region removes 13 Y genes completely and 10 Y genes partially with more copies located proximal and distal to the *AZFb* deletion interval (Vogt, 2004). Usually, complete *AZFb* deletions are associated with azoospermia manifested by the arrest of meiosis and maturation at the spermatocyte and spermatic stages of male germ-cell differentiation (Vogt *et al*., 1996; Krausz *et al*., 2000; Kamp *et al*., 2001; Affara, 2003; Vogt, 2004; Ferlin *et al*., 2007), although SCOS is not impossible. Likewise, combined *AZFbc* deletions are also a consequence of recombination between palindromic sequences. Although these deletions are large (~7.7 Mb), they do not include the distal portion of *AZFc* sequence (Repping *et al*., 2002).

2.4.3.1.3. The *AZFc* region

AZF deletions occur most frequently in the *AZFc* region with an estimated population frequency of approximately 1 in 4000 males (Vogt *et al*., 1996; Saxena *et al*., 2000; Yen, 2001; Kuroda-Kawaguchi *et al*., 2001; Oates *et al*., 2002; Skaletsky *et al*., 2003; Tyler-Smith and McVean, 2003; Repping *et al*.,

2003; Machev *et al*., 2004; Nathanson *et al*., 2005; Fernando *et al*., 2006). *AZFc* region is composed entirely of palindromic direct and inverted ampliconic repeats of considerable unit length (115-678 kb) that only differ by ~1nt per 3,000 bp. These rare subtle differences are called sequence family variants (SFVs) or single nucleotide variants (SNVs). This extraoridinary ampliconic structure of the *AZF* loci renders the region as a hot spot site for intrachromosomal ectopic homologous recombinations and subsequent spontaneous recurrent deletion errors (Skaletsky *et al*., 2003; Repping *et al.,* 2003; Machev *et al.*, 2004; Vogt, 2005a).

Kuroda-Kawaguchi (2001) together with his colleagues constructed a meaningful map of the *AZFc* region after sequencing the entire *AZFc* region. They found that *AZFc* consisted of three palindromes with six distinct ampliconic families. These amplicons have been given the names: turquoise (t), gray (gr), green (g), yellow (Y), blue (b), and red (r) (**Fig.2.5.**).

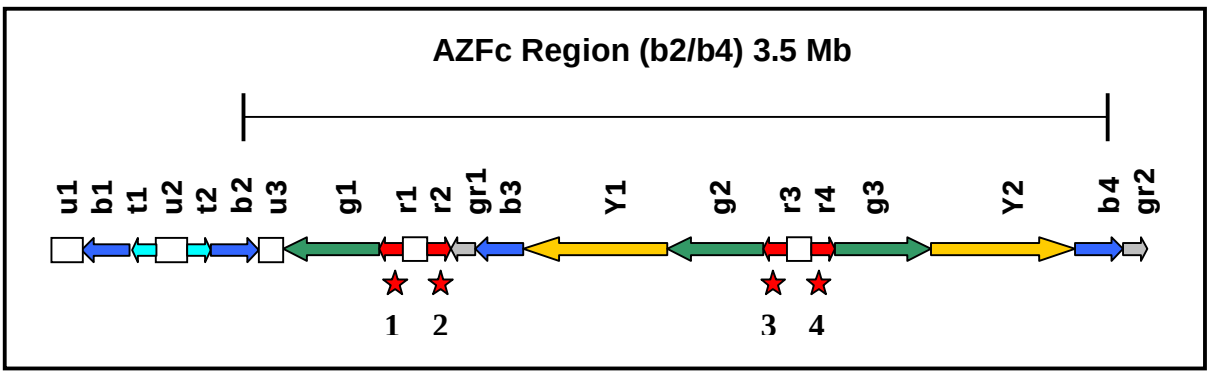

Figure 2.5. The reference ampliconic structure of the *AZFc* locus. *AZFc* amplicon structure is drawn according to the color code of Kuroda-Kawaguchi *et al*. (2001). Red stars numbered 1 to 4 show the location of the four *DAZ* genes.

As mentioned earlier, the *AZFc* region is delimited by two 229 Kb amplicons, proximal and distal, termed b2 and b4 (b for blue), respectively (see **Fig.2.5.**). Most of the *AZFc* deletions were between these two amplicons as witnessed by Kuroda-Kawaguchi *et al*. (2001) which spans approximately 3.5 Mb and is mapped to the deletion intervals 6D-6F, which removes all of the *AZFc* locus. The association of b2/b4 complete *AZFc* deletions (also called classical *AZFc* deletion) with spermatogenic failure is well established as the observed phenotype range from azoospermia to severe

oligozoospermia (sperm count <5M/ml) to oligozoospermia (sperm count 5-20 M/ml) (Ferlin *et al*., 2006; Repping *et al*., 2002; Foresta *et al*., 2001; Moro *et al*., 2000).

It was reported that patients with *AZF*c deletions and oligozoospermia will be exposed to a progressive decrease in their sperm counts overtime, as a result of spontaneous progressive regression of the germinal epithelium. This is why it is recommended for these patients to cryopreserve their semen for latter use when diagnosed avoiding the risk of becoming completely azoospermic or to avoid future more invasive techniques such as TESE/ICSI (Girardi *et al*., 1997; Simoni *et al*., 1997; Chang *et al*., 1999; Saut *et al*., 2000; Calogero *et al*., 2001; Dada *et al*., 2003; Ferlin *et al*., 2007), although this was discordant with Oates *et al*. (2002) findings who claimed that sperm production appeared to be stable over time in Y chromosome *AZFc* microdeleted patients.

Almost all b2/b4 *AZF*c deletions are essentially indentical in molecular extension, however, individuals carrying these microdeletions show variable grades of testicular failure, ranging from severe hypospermatogenesis, to SGA, to SCOS, although the former is the usual finding in testicular biopsies (Vogt *et al*., 1996; Oates *et al*., 2002; Arredi *et al*., 2007). It is of importance to know that mature spermatozoa (residual spermatogenesis) can generally be recovered from testicular tissue biopsies of azoospermic patients with *AZF*c deletions, whereas this usually is not the case in patients with complete *AZF*a or *AZF*b deletions (Saut *et al*., 2000; Vogt, 2005b). Consequently, TESE for ICSI treatment is not recommended for patients with complete *AZFa* or *AZFb* deletions, because usually they are unsuccessful (Krausz *et al*., 2000; Choi *et al*., 2004).

Genes within the *AZFc* region are present in multiple copies. The region includes 12 genes and transcription units each present in a variable number of copies making a total of 32 copies (Simoni *et al*., 2004). Candidate genes within the *AZFc* region include 4 copies of the *DAZ* (see below) , 3 copies of *BPY2* (Basic Protein on Y chromosome,2), and two copies of *CDY1* (*CDY1a* and *CDY1b*; Chromodomain protein, Y chromosome 1) (Reijo *et al*., 1995; Yen *et al*., 1998; McElreavey *et al*., 2000; Kuroda-Kawaguchi *et al*., 2001). The strongest candidate among them, in which its deletion is responsible for

the *AZFc* phenotype, is the *DAZ* gene family which encode putative RNA-binding proteins, that are exclusively expressed in the male germ cells, specifically in the spermatogonia and early primary spermatocyte (Menke *et al*., 1997; Saxena *et al*., 2000; Moro *et al*., 2000; Kuroda-Kawaguchi *et al*., 2001; Repping *et al*., 2002; Oates *et al*., 2002; Ferlin *et al*., 2005; Carvalho and Santos, 2005). Noteworthy, *DAZ* gene family have no X chromosome homolog, but surprisingly have two autosomal homologs, *DAZL1* (*DAZ* like-autosomal 1) located on chromosome 3 short arm (3p24) and *BOULE* located on chromosome 2 long arm (2q33). *DAZL* is expressed exclusively in the germ cells where it encodes for a RNA binding protein (Foresta *et al*., 2001; Ferlin *et al*., 2006). Therefore, it is suggested by Foresta *et al*. (2001) that *DAZL1* may synergitically act in combination with *DAZ* during spermatogenesis. *DAZL1* allows a small degree of spermatogenesis to survive in the majority of *AZF*c deleted men (Silber *et al*., 1998); which may explain the residual spermatogenesis in many *AZFc* deleted men. *BOULE* is also exclusively expressed in the testis, where it regulates meiosis through controlling the translation of *CDC25* transcripts (Luetjens *et al*., 2004; Krausz and Degl'Innocenti, 2006b; Kostova *et al*., 2007).

There was a substantial argument about how many copies of *DAZ* gene are present within the *AZFc* region. Reijo *et al*. (1995) claimed that there is only one copy of *DAZ*, while Glaser *et al*. (1998), Yen *et al*. (1998), and Moro *et al*. (2000) suggested that there are seven copies. However, Saxena *et al*. (2000) demonstrated that there are at least four *DAZ* copies (*DAZ1-DAZ4*) found in two clusters. The proximal *DAZ1* and *DAZ2* (situated in P2 palindrome) in one cluster, and the distal *DAZ3* and *DAZ4* (situated in the large P1 palindrome) in a second cluster, where the two genes in each cluster are arranged in two head-to-head clusters in amplicons r1-r4. This model was widely accepted and can explain why there is no point mutation events were detected within the *DAZ* genes, because if one is mutated the others will, of course, compensate (Ferlin *et al*., 2005; Lepretre *et al*., 2005; Fernandes *et al*., 2006). Lin (2005) together with here colleagues characterized 82 males with unknown fertility status for *DAZ* copy number by employing a novel DNA-blot hybridization and FISH strategies. They found that most males have four *DAZ* genes, exactly as being described by Saxena *et al*. (2000). Importantly,

five out of these 82 males were found to harbor six *DAZ* genes. More importantly, two of these men were oligozoospermic, one fertile man, and two of unknown fertility status. The question that remained to be answered what is the effect of the extra *DAZ* genes on male fertility? Noteworthy, *DAZ* genes are preferentially involved in quantitative rather than qualitative sperm production. Moreover, it does not affect or impair the leydig cell function (Mulhall *et al*., 1997; Oates *et al*., 2002).

Recently, a substantial attention was given to the *CDY1* gene as being the second candidate gene within the *AZFc* region, which encodes a histone acetyltransferase (Vogt, 2005a). This gene is specifically expressed in the testis, which may signify its importance in spermatogenesis. *CDY1* is present in two copies, *CDY1a* and *CDY1b*, within the AZFc region, one being embedded within the *DAZ* cluster, and the other is found within the distal *AZFc* region (Yen, 1998; Foresta *et al*., 2001; Machev *et al*., 2004; Giachini *et al*., 2005). Likewise *DAZ*, *CDY1* has an autosomal homolog on human chromosome 13, called *CDYL*, in contrast to the *DAZ* autosomal homologs, this gene is expressed ubiquitously. (Foresta *et al*., 2001; Vogt, 2005a; Krausz and Degl'Innocenti, 2006b).

2.4.3.1.4. Current modified classification of *AZF* region deletions

After the genomic human Y DNA sequence has been completed (Skalestsky *et al*., 2003), it revealed the real genomic structure and organization of this extraordinary chromosome, the most interesting prominent features being the presence of eight massive palindromes (P) within the Yq, as described earlier. Moreover, and as mentioned before, most of the *AZF* microdeletions are generated through intrachromosomal homologous recombinations between amplicons organized into palindromic structures showing a nearly identical sequences. The original classification in the *AZF* regions (a,b and c) proposed by Vogt *et al*. (1996) was therefore modified according to the mechanism of deletion in: *AZF*a, P5/proximal P1 (previously known as *AZF*b), P5/distal P1 (previously known as *AZF* bc*),* P4/distal P1 (also *AZF*bc), and b2/b4 *AZF*c (**Fig.2.6.**)[Repping *et al*., 2002].

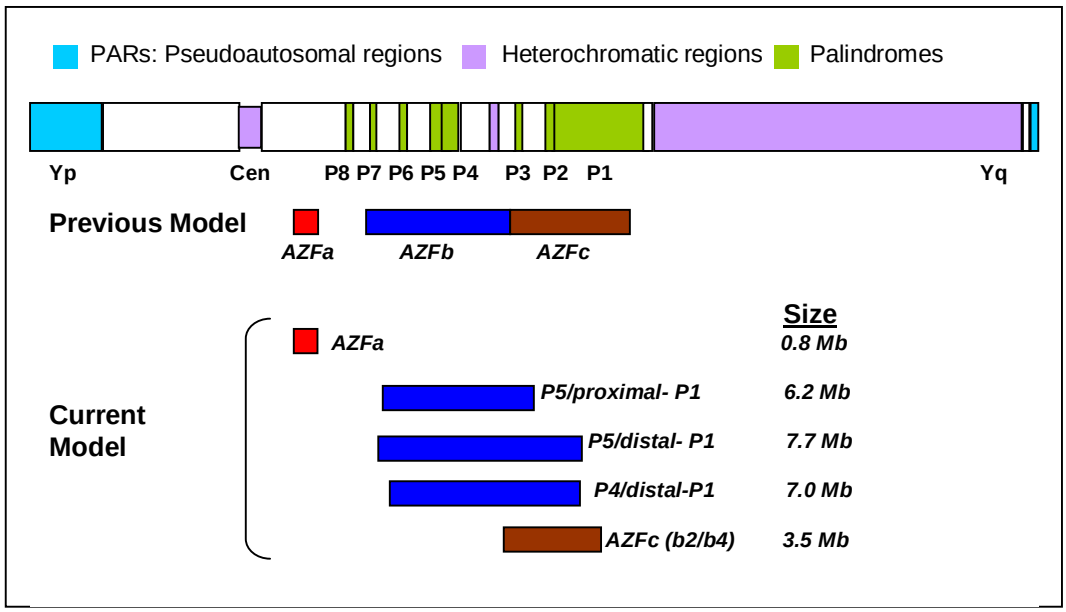

Figure 2.6. Previous model of recurrent, interstitial Y-chromosome deletions that cause infertility in men (Vogt *et al*. 1996) contrasted with current model (figure not in scale) (Adapted from Repping *et al*. 2002).

2.4.3.1.5. Incidence of *AZF* loci microdeletions

The incidence of Yq microdeletions in azoospermic and oligozoospermic infertile patients varies between studies, from 0% (Tzschach *et al*., 2001) to 55% (Foresta *et al*., 1998) (see **Table 2.1.**). This variability could be attributed to all of the following: (1) Patient recruitment, selection and inclusion criteria; (2) Molecular testing methodology in relation to choice and number of STSs used, their position and their reliability. Furthermore, the lack of rigorous testing of negative results (false deletions); (3) Population differences due to different Y chromsome haplogroups; (4) Inappropriate diagnosis, and (5) Size of the study, studies with low patient number report a higher frequency of deletions (Krausz and McElreavy, 1999a; Tzchach *et al*., 2001; Krausz and Degl'Innocenti, 2006b).

Table 2.1. Y-chromosome microdeletions reported from 1995-2007

Source	No. patients	No. deleted	total pat.(%)	No. STS
Reijo *et al*., 1995	89	12	(13.5)	84
Stuppia *et al.,* 1996	33	6	(18.2)	14
Reijo *et al*., 1996a	35	2	(5.7)	118
Vogt *et al*., 1996	370	12	(3.2)	76
Foresta *et al*., 1997	38	11	(29.0)	15
Pryor *et al*., 1997	200	14	(7.0)	85
Mulhall *et al.,* 1997	83	8	(9.6)	?
Girardi *et al.*, 1997	156	8	(5.1)	36
Van der Ven K, *et al*.,1997	204	2	(0.98%)	30
Foresta *et al*., 1998	18	10	(55.5)	29
Krausz *et al*., 1999b	134	3	(2.2)	7
Tse *et al.,* 2000	58	4	(6.9)	6
Martinez *et al.,* 2000	128	9	(7.0)	9
Tzschach *et al*., 2001	97	0	(0.0)	13
Peterlin *et al*., 2002	226	10	(4.4)	58
Bor *et al*., 2002	400	3	(0.75)	25
Ambasuhan *et al*., 2003	177	9	(5.1)	31
Dada *et al.,* 2003	83	8	(9.6)	6
Gruber *et al*., 2003	187	0	(0.0)	18
Asbagh *et al*., 2003	40	2	(5)	11
Saopedro *et al.,* 2003	60	4	(6.7)	13
Bush *et al.,* 2004	96	0	(0.0)	?
Athalye *et al*., 2004	100	12	(12)	18
El Awady *et* al., 2004	33	4	(12)	11
Swarna *et al*., 2004	70	9	(12.8)	5
Vicdan *et al*., 2004	208	19	(9.1)	?
Okutman-Emonts *et al*., 2004	71	4	(5.6)	18
Kihaile *et al*., 2005	113	7	(6.2)	24
Kihaile *et a*l., 2005	49	0	(0.0)	24
Medica *et al*., 2005	105	1	(0.95)	9
Hellani *et al*., 2006	247	8	(3.2)	19
Fernando *et al*., 2006	96	7	(7.3)	?
Omrani et al., 2006	99	24	(24.2)	18
Hsu *et al*., 2006	460	24	(5.2)	4
Pina-Neto *et al*., 2006	160	12	(7.5)	28
Mohammed *et al*., 2007	266	7	(2.6)	35
Imken *et al*., 2007	127	4	(3.15)	6

2.4.3.1.6. *AZF* microdeletions association with Y haplogroups

Many researchers in the field either hypothesize or assume that certain Y chromosome haplogroups may predispose or protect against specific type of Y chromosomal microdeletions. For example, Vogt (2005a) assumed that different Y haplogroups have variable *AZF*b and *AZF*c molecular structures. This viariability may cause sequence differences or amplicons orientation differences, changing the frequency of intrachromosomal recombinations. However, a limited number of studies in the literature aimed to find the possible association of certain Y chromosome haplogroups with Y

chromosome microdeletions. In particular, the literature represents only five studies, up to date, which have investigated this possibility, all failed, except the most recent one, in individuating possible significant association, **Table 2.2.** (Paracchini *et al*., 2000; Quintana-Murci *et al*., 2001; Carvalho *et al.*, 2003; Carvalho *et al*., 2004; Arredi *et al*., 2007).

Table 2.2. Y chromosome haplogroups and *AZF* deletions association studies

		No. of subjects analyzed			
Phenotype	**Population**	**Patients**	**Controls**	**Main finding**	**Reference**
AZF deletions	European	73	299	No association	Paracchini *et al*., 2000
AZF deletions	European	50	50	No association	Quintana-Murci *et al*., 2001
AZF deletions	Japanese	6	84	No association	Carvalho *et al*., 2003
AZF deletions	Israeli	9	-	No association	Carvalho *et al*., 2004
*AZF*c deletions	Italians	31	93	Association	Arredi *et al*., 2007

Only, Arredi *et al*. (2007) novel study signaled for an association between Y haplogroup E and Y chromosome microdeletions in North Italian population. Y haplogroup E was found to be more prone to rearrangements, and in particular to b2/b4 microdeletions, than other Y haplogroups. On the contrary, such association could also be questionable, since Kihaile *et al*. (2005) studied 49 infertile African males and none of them showed any Y chromosome microdeletions, provided that Africans have a very high incidence of Y haplogroup E (Underhill *et al*., 2000; Y-Chromosome Consortium, 2002).

More intriguing, Arredi with his coworkers (2007) also figured out that the Y haplogroup J have a much reduced frequency among Y chromosome *AZFc* microdeleted patient group (1/31; 3.2%) than in the fertile normal group (14/93; 15%). The authors also stated in their study that Y chromosome belonging to this Y haplogroup confer protection against Y chromosome microdeletion occurrence because of the molecular characteristics of the J haplogroup. In particular, it does not contain the LIPA4 element in the HERV15q2 block which is supposed to facilitate the homologous intrachromosomal recombination leading to *AZF*a deletion. It is of importance to know that haplogroup J reach high frequencies in Middle East populations (Krausz and Degl'Innocenti, 2006b).

For the timebeing, no conclusive results have yet been reached about the haplogroup role in association with Y microdeletions, and further studies in larger, well selected groups of subjects and geographical localization are indeed required to accredit such an attractive association with high certainity.

2.4.3.1.7. Association of FSH and Inhibin B levels with *AZF* deletions

Testicular endocrine function in patients with *AZF* deletions showed serum FSH levels above the mean value, but are these raised levels specifically associated with microdeletions? Kumar *et al*. (2006) found raised serum FSH levels in all of his patients who have severe depletion in germ cell function, irrespective of the presence or absence of *AZF* deletions, and the levels were indeed correlated with the severity of the testicular histology. On the other hand, Frydelund-Larsen *et al*. (2002) studied FSH levels in 16 *AZF* microdeleted patients and found accordant high FSH levels in the majority of them. On the contrary, Foresta *et al*. (2001) and Ferlin *et al*. (2007) found low FSH levels in the *AZF* microdeleted patients when compared to infertile men without deletion. Furthermore, Frydelund-Larsen *et al*. (2002) and Krausz *et al*. (2001) found a uniformly low plasma inhibin B in *AZF* microdeleted patients, but again this was discordant with the findings of Foresta *et al*. (2001) who reported a relatively high level of plasma Inhibin B in patients with *AZF* deletions. From the above discordant findings, we can conclude that hormonal data are not useful in predicting Y chromosomal deletion patients.

2.5. Partial *AZFc* deletions

The b2/b4 deletion takes out eight gene families including all members of the *DAZ* gene family, and carriers of such deletion have severe spermatogenic failure (Foresta *et al*., 2005; Lynch *et al*., 2005; Ferlin *et al*., 2006). Recent data suggested that other nonallelic homologous recombination events could occur between amplicons within the *AZFc* region (Vogt, 2005a). These recombination events can yield to different *AZFc* partial deletion patterns; i.e., characterized by loss of some, but not all, copies of all *AZFc* genes. At first sight, some of these deletions appear to have little or even no effect on male fertility, whilst others appear to carry an increased risk

of developing spermatogenic failure (Ferlin *et al*., 2006). These partial *AZFc* deletions are reported in azoo/oligozoospermic infertile men as well as in normozoospermic fertile men. (Machev *et al.*, 2004; Ferlin *et al*., 2005; Giachini *et al*., 2005; Lynch *et al*., 2005; Hucklenbroich *et al*., 2005; Carvalho *et al*., 2006; Ravel *et al*., 2006; Wu *et al*., 2007).

Different *AZF*c partial deletion patterns have been described, and thereafter termed (**Fig.2.7.**). They include gr/gr deletions (three subgroups: g1/g2, r1/r3, r2/r4) which remove 1.6 Mb, b1/b3 and b2/b3 (u3-gr/gr or g1/g3) deletions which remove 1.8 Mb, and other infrequent patterns (some are still not termed, but are characterized by the presence or absence of different *AZF*c region specific STSs) (Fernandes *et al*., 2002; Repping *et al*., 2003; Repping *et al*., 2004; Fernandes *et al*., 2004; Hucklenbroich *et al*., 2005; Ferlin *et al*., 2005; Giachini *et al*., 2005; Ferlin *et al*., 2006). Each partial deletion pattern could also be further divided into subtypes, and although the number of gene copies deleted is the same, the missing type is different (Giachini *et al*., 2005). *AZF*c partial deletions could be detected and characterized by performing FISH, southern blotting, *AZF*c specific STSs PCR, *DAZ*-specific SNVs, or gene dosage analysis, which also allows to clarify the underlying deletion mechanism (Ferlin *et al*., 2006).

It is still very debatable whether partial *AZFc* deletions affect spermatogenesis or not. The gr/gr deletion described by Repping *et al*. (2003) removes about half of the *AZFc* region and it is thought by the authors to be a risk factor for spermatogenesis failure, but its penetrance is far lower than that of deletions involving the entire *AZFc*. This suggests a polygenic dosage effect of multiple copy genes that might control spermatogenesis, as well as it may explain the reduction in sperm count. Ferlin *et al*. (2005) studied 337 infertile and 263 control normozoospermic fertile Italian men for partial *AZF*c deletions. They found 19 cases of partial *AZFc* deletions, 18 cases (16 gr/gr cases, 1 b2/b3 case, and 1 b3/b4 case) within the infertile group, and only one case (with gr/gr deletion) among the control fertile group. On the basis of the increased prevalence of partial *AZFc* deletions in their infertile men group, they suggested that such deletions represent a risk factor for male infertility, de Llanos *et al*. (2005) also reached the same conclusion on a study

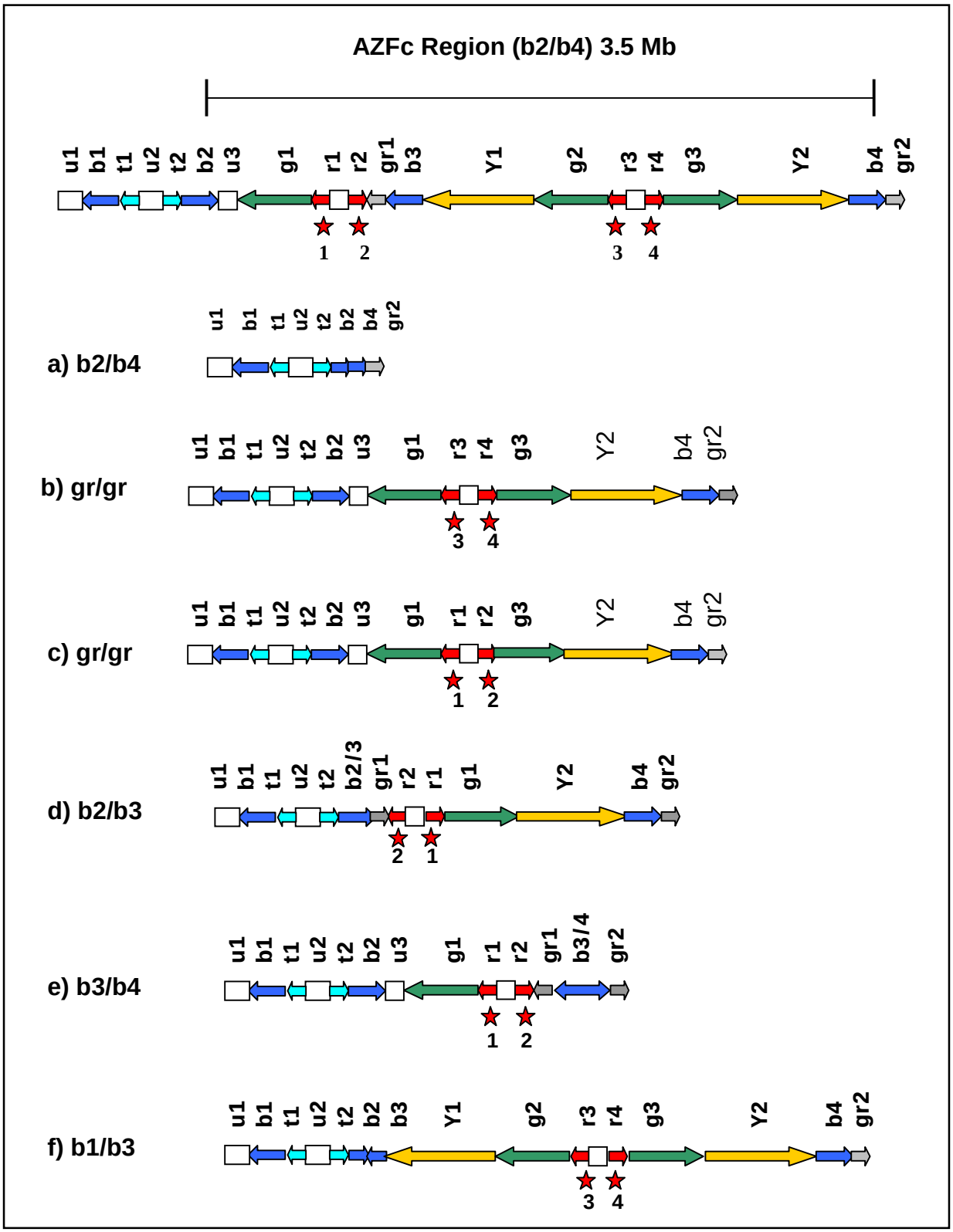

Figure 2.7. Schematic representation of *AZFc* structure before and after complete and partial deletions. Red stars numbered 1 to 4 show the position of the four DAZ genes; a) *AZFc* classical b2/b4 deletion; (b and c) gr/gr partial *AZFc* deletions; d) b2/b3 partial *AZFc* deletion; e) b3/b4 partial *AZFc* deletion; f) b1/b3 partial *AZFc* deletion (Adapted from Ferlin *et al*., 2005).

conducted on Spanish population. Furthermore, Ferlin *et al*. (2005) performed SNV deletion analysis of *DAZ* genes on these deleted cases, and concluded that *DAZ* genes are only removed in doublets, either *DAZ*1/*DAZ*2 or *DAZ*3/*DAZ*4. Most intriguing, they found that *AZF*c partial deletions removing *DAZ*1/*DAZ*2 doublets are associated with spermatogenic impairment. Also, Giachini *et al*. (2005) suggested on the basis of their work, that *AZFc* partial deletions are not specific for spermatogenic failure, but can be considered as a new risk factors for oligozoospermics. Both of the latter studies were done on Italians, and both studies found gr/gr deletions to be more prevalant than other partial *AZFc* deletions, and most importantly both studies where in concordance concerning the functional importance of *DAZ*1/*DAZ*2 doublet in male fertility; Fernandes *et al*. (2002) also found *DAZ1/DAZ2* gene doublet deletions only in men with severe oligozoospermia. However, Machev *et al*. (2004) found no difference in *DAZ*1/*DAZ*2 deletion frequency between infertile patient group and fertile control group, which appears to contradict this observation. Most interestingly, Giachini *et al*. (2005) also reported that deletion of the *CDY1a* copy was found only in the infertile group and was always associated with *DAZ*1/*DAZ*2 doublet deletion. A similar phenomenon has also been observed before in Machev *et al*. (2004) study performed on Mediterranean French population, which raises the importance of the *CDY1a* gene within the *AZFc* region in male fertility but this requires further confirmation.

Carvalho *et al*. (2006a), found no association between gr/gr deletions and male infertility in Brazilian white males, as also Zhang *et al*. (2006) and Wu *et al*. (2007) in Han-Chinese population, Fernando *et al*. (2006) in Sri Lankian population, and Imken *et al*. (2007) in Morroccan arab population.

Intristingly, Wu *et al*. (2007) found a strong association between b2/b3 subdeletions, which removes *DAZ3/DAZ4* doublet, with male infertility in Han-Chinese population. In this study, the prevalence of b2/b3 subdeletions in the infertile group (451 idiopathic infertile patients) was 8.9% compared to 3.2% in the normal control group (248 normal fertile males).

It is obvious from the previously mentioned studies that *AZFc* partial deletions could be compatible with male fertility, therefore they can be transmitted successfully from generation to generation. Consequently, they

are polymorphisms but not *de novo* rearrangements. This raises the question if they are associated with specific male lineage haplogroup/s. This sounds true, since Fernandes *et al*. (2004) identified that males with Y haplogroup N have consistently b2/b3 deletion (g1/g3 deletion) which removes *DAZ*3/*DAZ*4 and *BPY2.2*/*BPY2.3* doublets from the *AZFc* region without affecting their reproductive fitness; furtherance, proving the importance of *DAZ*1/*DAZ*2 doublet in male fertility and/or may suggest that these genes are not required for male fertility. Haplogroup association could also be confirmed by the findings of Carvalho *et al*. (2006b) who reported that about 30% of Japanese males belong to the Y haplogroup D2b, and all of them were found to have a fixed gr/gr deletion that did not alter their sustained fertility status. In addition, Repping *et al*. (2003) studied 12 men belonging to the Y haplogroup D2b and all of them were found to harbor gr/gr deleted Y chromosome. Although the two latter studies are contradicted on the effect of the gr/gr deletions on male fertility, nevertheless, they were in concordance concerning the strong association of the gr/gr deleted Y chromosome with the D2b Y chromosome. More recently, Zhang *et al*. (2007) found a fixed gr/gr deletion in all infertile patients as well as in normal fertile donors belonging to the Y haplogroup Q1. Furthermore, Machev *et al*. (2004) suggested that the effect of the gr/gr deletion would vary on Y chromosome of different Y chromosome haplogroups, and that most of gr/gr deletions are neutral variants and would not affect male fertility. Same conclusion has also been reached by Giachini *et al*. (2005) concerning the b2/b3 *AZFc* partial deletions.

It was proposed by Zhang *et al*. (2007) that candidates with partial *AZFc* deletions, as polymorphisms, will have greater risk than others to give complete *AZFc* deletion offsprings, causing male infertility.

Quite surprisingly, a novel study conducted on Han-Taiwanese (Lin *et al*., 2007) found that *AZF*c partial duplication is associated with an increased risk of impaired male fertility, but not the *AZF*c partial deletions. The authors attributed this finding to the increased expression of duplicated genes that may interfere with normal spermatogenesis.

For the moment, current literature showed that these *AZF*c partial deletion patterns still are subject to continuing intense debate and investigations, since contradictory and inconsistent, sometimes misleading,

findings were obtained with different recent studies. Moreover, published data cast doubts about *AZF*c partial deletions clinical relevance, consequently, it is still difficult to decide whether routine screening for partial *AZFc* deletions will be worthwhile (Repping *et al*., 2003; Machev *et al*., 2004; Fernandes *et al*., 2004; Hucklenbroich *et al*., 2005; Ferlin *et al*., 2005; Giachini *et al*., 2005; Lynch *et al*., 2005; de Llanos *et al*., 2005; Carvalho *et al*., 2006; Zhang *et al*., 2006; Ferlin *et al*., 2006; Ravel *et al*., 2006; Fernando *et al*., 2006; Wu *et al*., 2007; Lin *et al*., 2007; Zhang *et al*., 2007).

2.6. Diagnostic testing

2.6.1. Semen analysis

When male infertility is suspected, then the conventional semen analysis begins the workup. A carefully performed semen analysis is a highly predictive indicator of the functional status of the male reproductive hormonal cycle, spermatogenesis and the patency of the reproductive tract.

Semen analysis will determine the volume of the ejaculate, concentration of sperm, the percentage of sperm with normal motility (swimming ability) and the percentage of sperm with normal appearance (morphology). Abnormality of any of these factors can be associated with infertility. If no sperm are found (azoospermia), the semen is analyzed for the presence of fructose, a sugar that is produced by the seminal vesicles. Its absence indicates obstruction along the reproductive tract. The WHO normal ranges for semen analysis are shown in **Table 2.3**.

Table 2.3. WHO-1999: standard values for semen analysis

Semen Characteristic	Normal
Volume (ml)	≥2
pH	7.2-8.0
Sperm Concentration (M/ml)	≥20
Total Sperm Count (M/ejaculate)	≥ 40
Motility	≥50% with progressive motility or 25% with rapid motility within 60 min after ejaculation
Morphology (% normal)	≥ 14% of normal shape and form
Viability	≥ 50% of spermatozoa
WBC (M/ml)	< 1.0
Immunobead test (IBT)	<50% spermatozoa with adherent particles

2.6.2. Endocrinological evaluation

Most cases of male infertility are non-endocrine in origin. Routine evaluation of hormonal parameters is not warranted unless sperm density is extremely low or there is clinical suspicion of an endocrinopathy. The incidence of primary endocrine defects in infertile men is less than 3%. Such defects are rare in men with a sperm concentration of greater than 5 M/ml. When an endocrinopathy is discovered, however, specific hormonal therapy is often successful. A low testosterone level is one of the best indicators of hypogonadism of hypothalamic or pituitary origin. Low LH and FSH values concurrent with low testosterone levels indicate hypogonadotropic hypogonadism. Elevated FSH and LH values help to distinguish primary testicular failure (hypergonadotropic hypogonadism) from secondary testicular failure (hypogonadotropic hypogonadism). Most patients with primary hypogonadism have severe, irreversible testicular defects. On the other hand, secondary hypogonadism has a hypothalamic or pituitary origin and infertility may be correctable. Elevated FSH levels are usually a reliable indicator of germinal epithelial damage and are usually associated with azoospermia or severe oligozoospermia (sperm count < 5.0 M/ml), depicting significant and usually irreversible germ cell damage. In azoospermic and severely oligozoospermic patients with normal FSH levels, primary spermatogenic defects cannot be distinguished from obstructive lesions by hormonal investigation alone. Therefore, scrotal exploration and testicular biopsy should be considered. An elevated FSH level associated with small atrophic testes implies irreversible infertility, and a biopsy is not warranted.

The diagnostic value of prolactin measurement is extremely low in men with semen abnormalities unless these are associated with decreased libido, erectile dysfunction, and evidence of hypogonadism. Prolactin measurement is warranted in patients with low serum testosterone levels without an associated increase in serum LH levels.

Recently, plasma Inhibin B has been included in the male infertility workup especially in those patients with nonobstructive azoospermia. Inhibin B is secreted by sertoli cells in response to FSH, and is the major feedback regulator of FSH secretion in man. Plasma Inhibin B levels are closely

inversely correlated with FSH levels and may be regarded as sertoli cell function marker. In azoospermic patients of nonobstructive type, viable sperm extractions were successful in all patients with Inhibin B levels of greater than 80 pg/ml, irrespective of their FSH, LH, or testosterone levels (Lockwood, 2004).

2.6.3. Conventional cytogenetics

Conventional cytogenetics (karyotyping) describes the chromosomal constitution. It is based on the recognition of metaphasic chromosomes according to their specific banding patterns. The most widely used cytogenetic staining is G-banding. G-banding is still an important routine method since it gives a comprehensive overview of genetic alterations.

2.6.3.1. Basic cytogenetic procedures

Conventional cytogenetic studies require cells that are actively dividing. Specimens that contain proliferating cells are bone marrow, lymph nodes, solid tissues and chorionic villi. Peripheral blood lymphocytes require the addition of a mitotic stimulant (e.g. PHA, pokeweed) for the cells to undergo mitosis (stimulated culture). Chromosomes can be distinguished individually under the light microscope at metaphase. The choice of specimen for chromosomal analysis depends on clinical indication of the patient. In infertile male patients, which are our concern, the specimen of choice is peripheral blood sample.

2.6.3.1.1. Specimen collection and handling

Human peripheral blood specimen must be collected into sterile tubes containing preservative-free sodium heparin. Specimen should be transported immediately at room temperature to the laboratory. If delay is unavoidable, then specimen/s should be stored at 4ºC for no more than 3 days (Rooney, 2001).

2.6.3.1.2. Culture media

Specimens for cytogenetic studies are grown in aqueous growth media, a variety of media are available, which include RPMI 1640, McCoy's 5A, MEM, and Ham's F10. Suspension culture media are balanced salt solutions containing salts, glucose, and buffering system to maintain the proper pH. The culture media have to be supplemented with L-glutamine, fetal bovine serum (FBS) and antibiotics. L-Glutamine is an amino acid essential for cell proliferation. Serum is essential for good cell growth. About 10-30% FBS is usually added to the culture medium. Penicillin and streptomycin are bacterial inhibitors commonly used in culture media to retard the growth of Gram positive and Gram negative microorganisms, respectively. Mitotic stimulants such as PHA (phytohemagglutinin: a T lymphocyte mitotic stimulant) is added to the culture media to stimulate cell proliferation. Blood samples which consist of free-floating cells can be cultured in sterile centrifuge tubes or tissue flasks. Most media are commercially available as powder and as concentrated or ready-to-use aqueous media.

2.6.3.1.3. Specimen preparation for culture

Whole blood can be added directly to the culture medium or the white blood cells can be separated from the other blood elements (especially RBCs) and used to inoculate the culture medium. Separation of the white cells can be accomplished by centrifuging the sample or allowing it to rest undisturbed until the blood settles into three distinct layers. The lowest layer consists of the heavier red blood cells, the top layer consists of plasma, and the narrow middle layer, the buffy coat consists of the desired white cells, which can be removed and resuspend in culture media. Some cytogenetic practitioners add 0.85% ammonium chloride to lyse RBCs (Verma and Babu, 1995).

2.6.3.1.4. Cell harvest

After the cell cultures have been grown for 3 days, the metaphase cells are harvested. Harvesting involves collection of dividing cells at metaphase, hypotonic treatment, fixation, the placement of chromosomes on glass slides, so that they may be stained and microscopically examined. A mitotic inhibitor, colcemid that is an analogue of colchicine is usually used to obtain an

adequate number of cells at metaphase (increasing the mitotic index, MI). Colcemid binds to the protein tubulin, obstructing formation of the spindle fibers or destroying those already present. This prevents the separation of the sister chromatids in anaphase, thus collecting the cells at metaphase. Exposure time to colcemid is important. A longer exposure results in more metaphases being collected, but chromosomes will be shorter because they continue to condense as they progress through metaphase. Longer chromosomes are generally preferred for cytogenetic studies.

Hypotonic solution is added to the cells after exposure to colcemid. Water enters the cell by osmosis, thus causing the WBC cells to swell and RBCs to explode. This is critical for adequate spreading of the chromosomes on the slide. Timing is critical, too long an exposure will cause the cells to burst and too short an exposure will cause clumping of chromosome spreads. Examples of hypotonic solutions are 0.075M potassium chloride (KCl), 0.8% sodium citrate, diluted balanced salt solutions, and mixtures of KCl and sodium citrate. Morphology of the chromosomes is affected by the type of hypotonic solution used.

Fixative containing three parts of absolute methanol to one part glacial acetic acid (modified Carnoy's solution) is used to stop the action of the hypotonic solution and to fix the cells in the swollen stage. The fixative also lyses any remaining RBCs present in the sample, furthermore it ensures the removal of cytoplasm together with cell debris. The fixative must be prepared fresh before use because it absorbs water from the atmosphere. The fixed cell suspension is dropped onto glass slides. The concentration of the cell suspension can be adjusted to achieve optimal results. A good slide preparation has sufficient number of metaphases that are not crowded on the slide, metaphases that are well spread with minimal overlapping of the chromosomes, and no visible cytoplasm. Increased temperature and humidity enhance chromosome spreading, whereas cooler temperature and lower humidity decrease it. Longer exposure to hypotonic treatment makes the cell more fragile and thus increases spreading, but an inadequate exposure can result in cells that are difficult to burst. Variables in slide preparation include the height from which the cells are dropped; the use of wet or dry slides; the use of cold, room temperature, or warm slides; the use

of steam; air- or flame-drying the slides; and the angles at which the slide and/or pipette is held. The slides are ‘aged/baked' for two days at 56-60 ºC or for 1-2 hour at 90 ºC to enhance chromosome banding. Chromosomes can also be ‘aged’ by brief exposure to UV light or to Microwave oven (Sole and Woessner, 1992).

2.6.3.1.5. G banding and staining

G banding techniques create bands along the length of the chromosome. This property facilitates the positive identification of the individual chromosome pairs and permits the characterization of structural abnormalities. Banding resolution is an estimate of the number of light and dark bands in a haploid set (number of bands visible on 23 chromosomes, one of each autosomal pair and the X chromosome). The minimum estimate is about 400 bands. Well-banded, moderately high-resolution metaphases are usually in the 500-550 band level, and prometaphase cells can achieve a resolution of 850 or more bands (high resolution banding). High resolution banding facilitates a better karyotype analysis and more precise designation of breakpoints and subtle chromosomal abnormalities.

GTG banding is one of the several G-banded techniques and the most common. The ‘aged’ slides are treated with the proteolytic enzyme trypsin and stained with Giemsa. Besides Giemsa stain, Wright's or Leishman stain can be used. A series of alternate light and dark bands will be produced along the length of each chromosome. These banding patterns are the barcodes which enable the positive identification of each chromosome. The dark bands are A-T rich, late replicating, heterochromatic regions of the chromosomes, whereas the light bands are G-C rich, early replicating, euchromatic regions. The G-light bands represent the most active regions of the chromosomes compared to the G-dark bands which contain relatively few active genes. G-banding is routinely used for the clinical evaluation of human chromosomes in most laboratories. Its permanent nature (in comparison to Q banding) facilitates a thorough microscopic analysis.

2.6.3.1.6. Karyotyping automation

Labor intensive is the remarkable disadvantage of conventional cytogenetic techniques. Instruments such as robotic harvesters, environmentally controlled drying chambers, and computerized imaging systems have been developed to assist the cytogenetic laboratory in sample preparation and chromosome analysis. The Cytogenetics Laboratory at the Islamic University uses the computerized imaging system since 2005. Except for the imaging system, the other techniques for cytogenetic studies at the Islamic University cytogenetic laboratory are all virtually performed manually.

The traditional method of karyotyping involves photomicroscopy after the location of suitable metaphases. A camera is attached on top of the microscope to take photographs of the metaphase spread. The film and photographs are processed in a dark room. The metaphase spreads are cut and paired, then stuck on a karyotype card. Karyotyping by this procedure is time consuming. The process now has been superseded by a computerized imaging system. An image acquisition subsystem can be used to capture the metaphase spreads. The subsystem consists of a microscope camera adapter, a charged couple device (CCD) camera, a frame grabber and image capture software. Dedicated cytogenetic imaging software can perform karyotyping; banding analysis and ideogram display are all now commercially available. At the Islamic University-Gaza cytogenetic laboratory, the computerized imaging system used is the Applied Spectral Imaging system. Metaphase spreads are captured in the digital form by the capture station. The image is then transferred to the system workstation where karyotyping is performed. Karyotyping is performed semi-automatically. The cytogenetic practitioners have to check the karyotypes manually to ensure that the chromosomes are correctly paired. A printer produces a hardcopy of the metaphase spread and karyotype. The images consisting of metaphase spreads and karyotypes are stored as digital files on the optical disks.

Karyotyping is done by following the guidelines laid down by the international system for human chromosome nomenclature (ISCN, 1995), which is the definitive guide to terminology and writing of the karyotype. All metaphase spreads should be analyzed, and at least two karyotypes should

be prepared. If more than one cell line is present (i.e., mosaics), at least one karyotype must be prepared from each cell. The resolution should be at least 400 bands. When there are less than 20 analyzable cells and an abnormality has been detected, the number of abnormal and normal cells (if any) is reported. When fewer than 20 cells can be examined and an abnormality is not detected, the number of cells studied is reported and additional procedures (FISH, molecular studies) may be recommended if clinically appropriate.

2.6.4. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was invented in 1985 by Kary Mullis at Cetus Corporation. The PCR can amplify a small segment of DNA over a billionfold. The method uses a pair of oligonucleotide primers, each about 15 to 50 base pair (bp) in length designed to hybridize to opposite strands of DNA at sites of several kilobases (kb) apart. In the presence of deoxyribonucleotide triphosphates (dNTPs), DNA polymerase, and buffer the oligonucleotides prime the synthesis of DNA on the DNA template. The PCR is based on the repetition of a number of cycles, each cycle comprising of a set of 3 steps, heat denaturation, annealing of primers, and primer extension, all conducted in succession under different and controlled temperature conditions. In heat denaturation, the double stranded DNA (template) is denatured by heating to a high temperature that causes the two strands to dissociate. Annealing of primers, since the primers are present in excess over the DNA templates, the formation of the primer-template complex will be favored over the reassociation of the two DNA strands when the temperature is lowered. Primer extension (amplification step), the third step is the synthesis of a strand of complementary DNA, through the 5' to 3' extension of each annealed primer. The 3 steps can be accomplished automatically with a thermal cycler. Usually 25 to 35 or more cycles are performed. In each cycle, the DNA fragments synthesized in the previous cycles becomes templates for the new DNA synthesis. The DNA fragments accumulate exponentially at a rate of 2^n, where n equals the number of cycles performed. The PCR products are DNA of uniform length that spans the distance between the sites of oligonucleotide priming. The ends of each amplified DNA fragment (also

known as target sequence product or amplicon) have nucleotide sequences that correspond to that of the oligonucleotide primers. However, the exponential amplification is not an unlimited process. A number of factors act against the process being 100% efficient at each cycle. Their effect is more pronounced in the latter cycles of PCR. The amount of enzyme becomes limiting after 25-30 cycles of PCR due to molar target excess. The enzyme activity is also reduced due to thermal denaturation. As the concentration of the target strands increases, it competes with primer annealing by reannealing of target strands. The PCR can amplify DNA fragments that are smaller than 5 kb. For large DNA fragments, PCR is inefficient due to the difficulty of synthesizing DNA of sufficient length from one primer to serve as a template for the other primer in the next amplification cycle. The PCR has found many applications such as the diagnosis of disease states, species identification, and detection of viral or bacterial infection, prenatal diagnosis, and forensic sciences.

The basic components of the PCR are the DNA template, DNA polymerase, dNTPs, primers, magnesium chloride ($MgCl_2$), and buffer. The various reagents are available commercially and some come in kit form.

Confirmation of PCR products are usually resolved by ethidium bromide stained agarose gel electrophoresis and viewed under ultraviolet (UV) light. PCR products can also be confirmed by restriction endonucleases (this depends on the presence of a suitable restriction site within the amplified sequence). PCR may yield false-positive reactions. Either false priming can occur on the genomic DNA or on the primers themselves, resulting in amplified fragments containing irrelevant DNA. The amplified product can be further confirmed by Southern Blot analysis of the PCR products followed by hybridization with the appropriate DNA probes, or a second round of PCR can be performed on the amplified DNA fragments using ‘nested’ oligonucleotide primers that correspond to such internal sequence (nested PCR).

Y chromosome microdeletions can be detected by PCR using the different STSs distributed along the Y chromosome, these STSs are distributed every ~30 kb (Aknin-Seifer *et al*., 2004) and enable detection of small deletions in the *AZF* regions that were undetectable with classical cytogenetic techniques.

Chapter Three

MATERIALS and METHODS

3.1. Study population

In the present study, 86 consecutive infertile Palestinian males residing in Gaza Strip with nonobstructive azoospermia (zero sperm count), cryptozoospermia (sperm count <0.1 M/ml), severe oligozoospermia (sperm count >0.1 and < 5 M/ml), or oligozoospermia (sperm count 5-10 M/ml) were selected and recruited from assisted reproduction centers and private infertility clinics, during the period from June 2006 to August 2007. Patients presented with primary infertility and having sperm counts less than 10 M/ml on 2 consecutive occasions, at least, were our candidates for the present study. One patient was omitted (case# 21) from our study because his azoospermia was later been found to be due to obstruction. A scheduled interview for each patient have been arranged to collect demographic data, past medical history, including history of chemotherapy, radiotherapy, varicocele, and cryptorchidism, and habits concerning smoking, alcohol consumption, and drug intake. Semen analysis results were collected for each patient. In addition, hormone profile (FSH, LH, total testosterone, prolactin, and Inhibin B) and histological testicular biopsy reports were collected, whenever available.

The proven fertility control group consisted of 30 Gazian men with proven fertility, defined as naturally conceiving at least one child. DNA extracted from there ethylenediaminetetraacetic acid (EDTA) blood samples were only analyzed for the presence of *AZFc* partial deletions.

Testing for the origin of the *AZFc* partial microdeletions (i.e., inherited or occurring *de novo*) was only possible for 4 patients, because blood from patients' fathers or brothers was not always available. DNA extracted from the available relatives EDTA blood samples were only analyzed for the presence of *AZFc* partial deletions.

3.2. Materials

3.2.1. Chemicals and reagents

Chemicals and reagents used in this study are shown in **Table 3.1**. All chemicals were of analytical and molecular biology grade.

Table 3.1. Chemicals and reagents used in the present study

Reagent	Supplier
Wizard® Genomic DNA Purification Kit	Promega, Madison, WI, USA
PCR Master Mix	Promega, Madison, WI, USA
Go Taq® Green Master Mix	Promega, Madison, WI, USA
Agarose Gel	Promega, Madison, WI, USA
Nuclease free water	Promega, Madison, WI, USA
DNA molecular size marker (Ladder), 100bp	(1) Promega, Madison, WI, USA (2) BioLab, New England
PCR primers	(1) Operon (Germany) (2) IDT (USA)
Ethidium Bromide (EtBr) 10mg/ml	Promega, Madison, WI, USA
COLCEMID SOLUTION (10 µg/ml)	Biological Industries, Israel
POTASSIUM CHLORIDE SOLUTION (0.075M)	Biological Industries, Israel
Trypsin EDTA (10x)	Biological Industries, Israel
HANKS' BALANCED SALT SOLUTION Without Calcium and Magnesium	Biological Industries, Israel
METHANOL, Absolute, Acetone Free	Frutarom, Israel
GLACIAL ACETIC ACID	Chembal Company, Gaza Strip
PCR Loading Solution (Bromophenol Blue) (6x)	0.25% Bromophenol blue 40% (w/v) sucrose in water
Tris-Acetate-EDTA (TAE) (50x)	2M Tris base (pH 8), 1M Glacial Acetic Acid, 0.05M EDTA (pH 8)
Leishman Stain	Sigma, USA
Culture grade water	Biological Industries, Israel

3.2.2. Culture media

Ready made RPMI1640 suspension medium supplemented with L-glutamine, fetal bovine serum (FBS), antibiotics, and phytohemagglutinin was used for culturing peripheral blood lymphocytes (PERIPHERAL BLOOD KARYOTYPING MEDIUM (RPMI 1640) with phytohemagglutinin**,** Biological industries, Israel).

3.2.3. Disposables

The major disposables used in this study are listed in **Table 3.2.**

Table 3.2. Major disposables used in the present study

Item	Item
Falcon Screw Cap Sterile Tubes 15 ml	Microfuge tubes - 1.5 ml capacity
PCR Microfuge tubes for PCR - 0.2 ml capacity	Gauze
Standard size precleaned glass slides	Disposable Gloves
Disposable Micropipette tips to deliver 1-5000 µL	EDTA Tubes
Sodium Heparin Tubes	Weighing cups

3.2.4. Equipment

All experiments of this study were done at the Islamic University of Gaza-Genetics Laboratory. The major equipment that were used are listed in **Table 3.3**.

Table 3.3. Major equipment used in the present study

Instrument	Manufacturer
Thermocycler	Eppendorf, Germany
	Biometra, Germany
Electrophoresis chambers/tanks	BioRad, USA
Electrophoresis power supply	BioRad, USA
Microcentrifuge	Sanyo, UK
Microwave	LG, Korea
CO_2 Incubator	N-BIOTEK, INC, Korea
Bench top Centrifuge	LWScientific, USA
Bench top Cooled Centrifuge	Centurion Scientific LTD, UK
Water Bath	*P*SELECTA, Spain
pH Meter	Sartorius, Germany
Phase Contrast Microscope	MEIJI Techno Co. Ltd, Japan
Binocular Microscope	Olympus, Japan
Trinuclear Microscope	Olympus, Japan
Freezer - 20ºC	LG, Korea
Freezer -70ºC	Napco, Czech Republic
Documentation System: Vision	SCIE-Plas Ltd, UK
Safety Cabinet	Heraeus, Germany
Automated Karyotyping System	Applied Spectral Imaging LTD, USA
Computer System	DELL, USA
Spectrophotometer	NanoDrop, USA

3.2.5. PCR oligonucleotide primers

All STSs PCR primers (indicated from 5`→ 3`) adopted in this study are shown in **Table 3.4.** Sense primers end with F (for forward), while antisense end with R (for reverse). All primer sequences were obtained from published literature.

Table 3.4. Sequences of the different primer sets adopted in the present study

STS	Primer Sequence	Product Size (bp)	Accession no.	Reference
ZFY-F	ACCRCTGTACTGACTGTGATTACAC	495		Simoni *et al*., 2004
ZFY-R	GCACYTCTTTGGTATCYGAGAAAGT			
SRY-F	GAATATTCCCGCTCTCCGGA	472	G38356	Simoni *et al*., 2004
SRY-R	GCTGGTGCTCCATTCTTGAG			
sY746-F	TTGACTGCTTATTCTACACAAGGC	216	G49213	Sun *et al*., 2000
sY746-R	CAGGGGAAATTGGGTTTT			
sY84-F	AGAAGGGTCTGAAAGCAGGT	326	G12019	Simoni *et al*., 2004
sY84-R	GCCTACTACCTGGAGGCTTC			
sY86-F	GTGACACAGACTATGCTTC	320	G49207	Simoni *et al*., 2004
sY86-R	ACACACAGAGGGACAACCCT			
DBY1-F	TATTGGCAATCGTGAAAGAC	277	G49468	Foresta *et al*., 2000
DBY1-R	TGCCGGTTGCCTCTACTGGT			
sY117-F	GTTGGTTCCATGCTCCATAC	261	G11996	Ferlin *et al*., 2003
sY117-R	CAGGGAGAGAGCCTTTTACC			
sY125-F	GGGATAGGGAAAGGGTACAA	200	G66531	Vollrath *et al*., 1992
sY125-R	CCGGGAGAAAAAAAACTGAA			
sY127-F	GGCTCACAAACGAAAAGAAA	274	G11998	Simoni *et al*., 2004
sY127-R	CTGCAGGCAGTAATAAGGGA			
sY131-F	ACATATCCCTTGCCACTTCA	143	G75620	Vollrath *et al*., 1992
sY131-R	TCAGGTACCTTCTGCCTGAG			
sY134-F	GTCTGCCTCACCATAAAACG	301	G12001	Simoni *et al*., 2004
sY134-R	ACCACTGCCAAAACTTTCAA			
sY152-F	AAGACAGTCTGCCATGTTTCA	125	G75623	Vollrath *et al*., 1992
sY152-R	ACAGGAGGGTACTTAGCAGT			
sY254-F	GGGTGTTACCAGAAGGCAAA	380	G38349	Simoni *et al*., 2004
sY254-R	GAACCGTATCTACCAAAGCAGC			
sY255-F	GTTACAGGATTCGGCGTGAT	123	G65827	Simoni *et al*., 2004
sY255-R	CTCGTCATGTGCAGCCAC			
sY272-F	GGTGAGTCAAATTAGTCAATGTCC	95	G65830	Reijo *et al*., 1995
sY272-R	CCTTACCACAGGACAGAGGG			
sY1191-F	CCAGACGTTCTACCCTTTCG	385	G73809	Repping *et al*., 2002
sY1191-R	GAGCCGAGATCCAGTTACCA			
sY1291-F	TAAAAGGCAGAACTGCCAGG	527	G72340	Repping *et al*., 2002
sY1291-R	GGGAGAAAAGTTCTGCAACG			

3.3. Methods

3.3.1. Blood collection

Approximately 5 ml venous blood were collected from each patient, and divided into two tubes, one containing sodium heparin for the cytogenetic testing, and the other contains EDTA for the molecular testing.

3.3.2. Cytogenetics/ GTG karyotyping

GTG karyotyping was performed by culturing patient's peripheral blood lymphocytes, from heparinized blood, in ready made RPMI 1640 complete aqueous culture media (Biological Industries, Israel) containing the mitogen phytohemagglutinin (PHA), L-glutamine, antibiotics (Penicillin and Streptomycin), and fetal calf serum; and according to the following procedure:

- Half milliliter heparinized blood was added to 5 ml of RPMI 1640 full medium in sterilized 15 ml falcon tube, mixed by inversion and incubated for 72 hours at 37ºC, 5% CO_2 in CO_2 incubator.
- After 72 hours, 100 µl Colcemid (10 µg/ml) was added and mixed well with subsequent incubation for 30 minutes at 37ºC.
- The cell culture tube was then centrifuged at 1200 rpm for 10 minutes and the supernatant was discarded leaving about 0.5 ml of supernatant remaining above cell pellet.
- Cell pellet was resuspended in the remaining medium and 8 ml of prewarmed 0.075 M KCl was added with continuous gentle agitation to ensure proper mixing.
- The cell culture tube was then incubated for 10 minutes at 37ºC in the incubator.
- Cells were collected by centrifugation as above described.
- Cells were then fixed by adding 10 ml of freshly prepared fixative (1 part glacial acetic acid with 3 parts absolute methanol), with gentle agitation with subsequent incubation for 10-15 minutes at room temperature.
- The cells were collected by centrifugation as above described.
- The fixation step was repeated for at least two extra times to ensure proper fixation and removal of debris.
- After the last centrifugation, the cells were resuspended in a small amount of fixative.
- One to two drops of fixed cell suspension were applied from a height to the center of a pre-cleaned standard size glass slide.
- The slides were then incubated at 56ºC in an oven for at least 2 days (extra baking time will usually give better results).

- The slides were then treated with 0.025% trypsin dissolved in Hank's balanced salt solution for 8-20 seconds.
- The trypsinized slides were then stained in 1.5% Leishman stain for 3-5 minutes and left to dry at RT.
- The slides were then examined under the microscope, and checked for chromosome bands.
- Good metaphase spreads were captured by the CCD camera, and were analyzed with the aid of Spectral Imaging software.

At least 20 metaphases (400-550 bands per genome) were analyzed for each patient, and 5 metaphases were karyotyped aided by the computerized imaging system (Applied Spectral Imaging LTD, USA). Karyotyping was performed by following the guidelines set by the International System for Human Cytogenetic Nomenclature (ISCN, 1995). In case of mosaicism the number of metaphases was increased to a total of 100 per analysis.

3.3.3. Extracting and purifying genomic DNA

Genomic DNA from patient and control EDTA blood samples was extracted and purified by using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), and adhering to the manufacturer protocol, which can be summarized as follows:

1- Three hundred µl of well mixed blood were added to 1.5 ml Microfuge tube containing 900µl of cell lyses solution (lyses both red blood cells and white blood cells), mixed gently by inversion, and the lysate was incubated at room temperature for 10 minutes. During the incubation period, the tube was periodically mixed (2-3 times) by gentle inversion.

2- The mixture was then centrifuged at 13,000 rpm for 20 seconds, and the supernatant was removed and discarded without disrupting the visible white pellet, then the white pellet was resuspended by vigorous vortexing (10-15 seconds).

3- Three-hundred µl nuclei lyses solution, which lyses the nuclear membrane of white blood cell nuclei, were added to the resuspended pellet with pipetting the solution 5-6 times. The solution by now should become very viscous; the samples with visible clumps should be incubated at 37ºC until the clumps are disrupted.

4- One-hundred protein precipitation solution, which precipitates nuclear and cytoplasmic proteins, were added to the nuclear lysate and they were mixed vigorously for 10-20 seconds by the vortex, then centrifuging at 13,000 rpm for 3 minutes at room temperature. A dark brown protein pellet should be visible.
5- The supernatant was then transferred to a clean 1.5 ml Microfuge tube containing 300µl isopropanol and was mixed gently by inversion until white thread-like strands of DNA form a visible mass, then centrifuged at 13,000 rpm for 1 minute. The DNA will be visible as a small white pellet.
6- The supernatant was discarded and 300 µl of 70% ethanol were added to the small white pellet and gently inverted several times to wash the DNA pellet, then centrifuged at 13,000 rpm for 1 minute.
7- The ethanol was aspirated carefully, and then the tube was inverted on clean gauze. The pellet was air-dried for 10-15 minutes.
8- The DNA pellet was rehydrated by adding 100µl DNA rehydration solution and incubated at 65ºC for one hour. The DNA pellet should be mixed periodically by gently tapping the tube.
9- Finally, the DNA was stored at 2-8ºC until PCR analysis is carried out.
10- The quality of the extracted DNA was monitored on ethidium bromide stained 2% agarose gels and the quantity of DNA (1 OD at 260 nm ~50 µg/ml) was measured by spectrophotometer (Nanodrop, USA) at 260 nm.

3.3.4. Molecular analysis

3.3.4.1. Primers (STSs)

Microdeletion analysis of the Y chromosome Yq *AZF* region involved two steps. In the first step, aimed at detecting *AZFa*, *AZF*b and *AZF*c complete microdeletions, a total of 13 previously published STSs (*AZF* loci) that are mapped at intervals 5 and 6 on the long arm of the Y chromosome were used (**Fig.3.1**); sY746, **sY84**, **sY86**, and *DBY1* for *AZFa*, sY117, sY125, **sY127**, sY131, and **sY134** for *AZFb*, and sY152, sY272, **sY254**, and **sY255** for *AZFc*. STSs shown in bold are all those recommended by the European Academy of

Andrology/European Molecular Genetics Quality Network (EAA/EMQN) guidelines, which suppose to detect over 90% of Y chromosome microdeletions, the other STSs are supplementary and were used to increase microdeletion detection rate. In addition, *SRY* (sex determination region of Y) gene and the X/Y homologous gene pair Zinc-Finger X (*ZFX*) and Zinc-Finger Y (*ZFY*) primers were designed to be used as positive internal controls to detect amplification failures in case a microdeletion was detected. The sequences of all primer pairs and the expected size of their products are shown in **Table 3.4**.

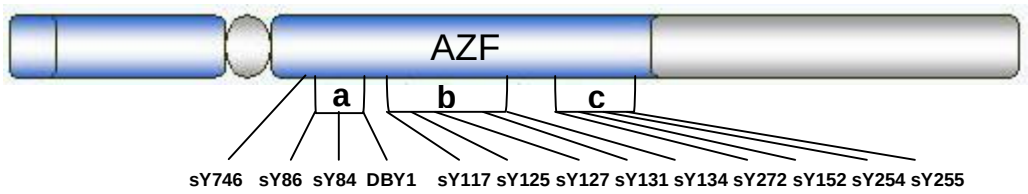

Figure 3.1. Relative physical positions of the STSs adopted in the study.

In the second step, when step one does not show any *AZF* deletion for any patient, we looked for *AZFc* partial deletions using sY1291, and sY1191 primer sets. The physical position of these STSs within the *AZFc* region is shown in **Figure 3.2**. The sequences of both primer pairs and their expected product sizes are also shown in **Table 3.4**.

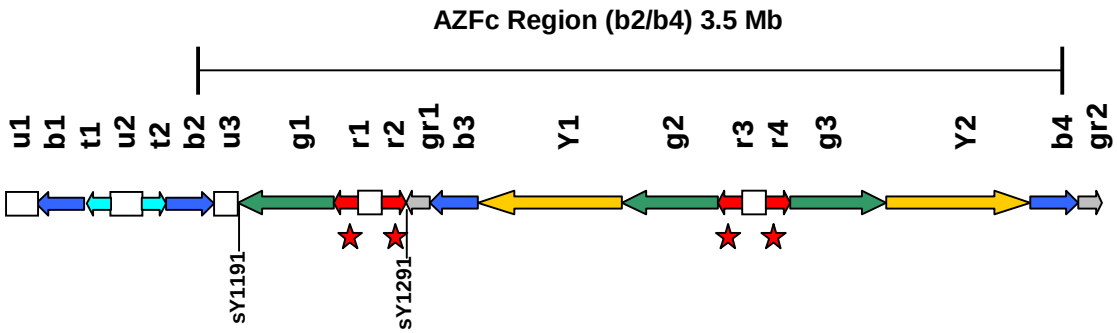

Figure 3.2. Physical positions of the adopted STSs that detect partial *AZFc* deletions.

3.3.4.2. Controls for microdeletions

Positive and negative controls were run concurrently with each patient sample. Female and proven fertile male DNA samples were used as negative and positive controls, respectively. Water instead of genomic DNA was used as blank to check for any DNA contamination.

3.3.4.3. Yq microdeletion analysis by PCR-based STS

PCR was carried out in a monoplex (single primer set, simplex) fashion for each primer set. PCR was carried out in 0.2 ml PCR Microfuge tubes in a 20 µl reaction volume containing: 2 µl template genomic DNA (100-200 ng), 10 µl PCR Master mix or Go Taq® Green Master Mix (Promega Madison, WI, USA), 1.5 µl (2 µmol) each primer, and nuclease free sterile distilled water to 20 µl. The amplification reaction was performed in a programmable thermal cycler. Amplification was started with an initial denaturation step at 94ºC for 15 minutes, followed by 35 sequential cycles each including 60 seconds denaturation at 94ºC, 60 seconds primer annealing at 57ºC and 60 seconds extension at 72ºC. The protocol was followed by a final extension step at 72ºC for 10 minutes and then cooling to 4ºC to be ready for electrophoretic detection.

For detecting *AZFc* partial deletions, we used the same reaction mixture and volume as above, but instead using different primer sets. The following PCR protocol was employed: 5 minutes initial denaturation (94ºC), followed by 35 sequential cycles of 30 seconds denaturation (94ºC), 45 seconds primer annealing (61ºC), and 45 seconds extension (72ºC), ended by an extension step of 7 minutes at 72ºC and then cooling to 4ºC to be ready for electrophoretic detection.

With a PCR product of the expected size, the reaction was regarded as positive for this STS. Negative PCR products (no amplification) of the same STS in 3 consecutive independent attempts were interpreted as deletions for this STS.

3.3.4.4. PCR products electrophoretic detection

The PCR yield product was added to the loading dye, mixed and run on a 2% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide in 1x Tris Acetate EDTA (TAE). In addition, a 100 bp DNA ladder was always run concurrently with each electrophoretic run to detect product sizes. After electrophoresis at 70 volts for 45 minutes, the results were visualized and documented using the UV transilluminator documentation system (Vision, Scie-Plas Ltd, UK).

3.4. Statistical analysis

In general, parametric statistics were used. Comparisons of qualitative data were carried out by means of Chi-square test (χ^2) in 2X2 contingency tables. While quantitative data were handled by means of unpaired Student's t-test for independent samples. Statistical analysis was performed using the statistical package SPSS for windows (version 12, SPSS Inc., Chicago, IL, USA) and Minitab version 14.12 statistical software. A *P* value of less than 0.05 was adopted to indicate statistical significance for each test.

Chapter Four

RESULTS

4.1. Patient data analysis

The general features and findings of our patient population are summarized in **Table 4.1**.

Table 4.1. General features and findings in the studied population

No. of patients (n)	85
Age (yrs)	30.49 ± 6.43 (18-52)[a]
Height (cm)	175.12 ± 7.25 (156-198)
Weight (kg)	78.65 ± 12.18 (58-115)
Duration of infertility (yrs)	6.3 ± 5.2 (0.0-29)[b]
Seminal fluid volume (ml)	3.0 ± 1.0 (1.0-5.2)
Patients with history of:	
Infertility	16 (18.8%)[c]
Alcohol consumption	01(1.18%)
Smoking	26(30.6%)
Varicocele	27(31.8%)
Cryptorchidism	07(8.2%)
Chemotherapy	None
Radiotherapy	None

[a] Mean ± SD (range)

[b] 3 of our patients were unmarried

[c] n(%)

4.2. Spermatogenic potential

Based on sperm count, our patient population was classified into four subgroups: azoospermic (59, 69.4%), cryptozoospermic (3, 3.5%), severe oligozoospermic (17, 20%), and oligozoospermic (6, 7.1%), **Table 4.2**.

Table 4.2. Classification of patients according to their sperm counts			
Sperm count category	# Patients	Sperm count range M/ml	%
Azoospermic	59	0.0	69.4
Cryptozoospermic	3	0.03-0.1	3.5
Severe Oligozoospermic	17	0.2-4.2	20
Oligozoospermic	6	6.0-10.0	7.1

4.3. Hormonal parameters data

Hormonal profile (FSH, LH, total testosterone, prolactin, and Inhibin B) data was not available for all patients, nor was complete hormone profile available for all patients. Serum FSH level was available for 79 patients, serum LH level was available for 72 patients, total testosterone level was available for 75 patients, prolactin level was available for 71 patients, while Inhibin B level was only available for 29 patients (25 azoospermics and 4 severely oligozoospermics). **Table 4.3** summarizes the available hormonal data for the patient population. **Table 4.4** summarizes the mean available hormonal values for each spermatogenic potential subgroup.

Table 4.3. Summary of the available hormonal values in entire patient population		
Hormone	# of patients	Mean ± SD (range)
FSH (mIU/ml)	79	16.96 ± 11.35 (0.0-43.0)[a]
LH (mIU/ml)	72	10.55 ± 6.62 (0.0-31.6)
Total Testosterone (ng/ml)	75	3.9 ± 1.61 (0.5-8.9)
Prolactin (ng/ml)	71	12.6 ± 5.47 (2.2-24.0)
Inhibin B (pg/ml)	29	55.3 ± 28.43 (17.3-160)

Table 4.4. Mean hormone profile values for each spermatogenic potential subgroup				
	Mean (range)			
	Azoospermics	Cryptospermics	Severely Oligospermics	Oligospermics
FSH	19.5 (0-43)	26.5 (22-31)	9.2 (2.2-26)	7.8 (4.5-12.4)
LH	10.5 (0-31.6)	15.3 (9-21)	10.8 (5.9-19.7)	8.7 (1.6-16.3)
Total Testosterone	3.8 (0.5-8.9)	3.4 (3-3.8)	4.2 (2.2-6.8)	4.1 (2.2-5.7)
Prolactin	12.1(2.2-24)	18.7 (n=1)	13.6 (4.9-21.6)	14.9 (8-20.8)
Inhibin B	52.1 (17.3-120)	21 (n=1)	82.9 (42-160)	ND[a]

[a] ND: No data available

Table 4.5. Hormonal t-test *P* values when comparing azoospermics with the other spermatogenic potential subgroups

Hormone	Cryptozoospermics	Severely oligozoospermics	Oligozoospermics
FSH	0.401	0.002 [a]	0.017 [a]
LH	0.355	0.894	0.537
Testosterone	0.743	0.484	0.734
Prolactin	0.230	0.408	0.225
Inhibin B	0.144	0.041 [a]	-

[a] Tagged *P* values indicate statistical significance (*P*<0.05)

As can be noted from **Table 4.5**, there is a statistically significant difference in FSH values between azoospermics and severely oligozoospermics (*P*=0.002), as well as between azoospermics and oligozoospermics (*P*=0.017). FSH values in azoospermics were higher than the other spermatogenic potential subgroups, except cryptozoospermics (*P*= 0.401). In addition, Inhibin values in azoospermics were lower than severely oligozoospermics (*P*=0.041), however it should be noted that only 4 severely oligozoospermic patients did the Inhibin B quantitation. While, there was no statistical significant difference in LH, total testosterone, or prolactin between the azoospermics and the different spermatogenic potential subgroups. We can observe that the FSH levels are in direct relationship with degree of spermatogenic failure.

4.4. Testicular histology

Only 46 testicular biopsy histological reports were available for the studied population. Forty-two reports were available for the azoospermic patient group, three for the cryptozoospermic patient group (cases# 52, 56, and 77), while only one (case# 60; sperm count 0.6 M/ml) for the severe oligozoospermic patient group. No reports were available for any patient in the oligozoospermic patient group.

Table 4.6 summarizes the results of these reports into Sertoli cell-only (SCO), spermatogenesis arrest (SGA) (although different patients show spermatogenic arrest at different stages of spermatogenesis, with no spermatozoa, but we collectively referred to them as SGA), and

hypospermatogenesis (HS). We observed the absence of SCO picture in the cryptozoospermic or severe oligozoospermic patients.

Table 4.6. Summary of results for the available testicular histology reports

Diagnosis	Total	Azoospermics	Cryptozoospermics	Severe Oligozoospermics
SCO	7	7	0	0
SGA	26	25	1	0
HS	13	10	2	1
Total	46	42	3	1

SCO patients have significantly higher FSH values than SGA patients (*P*=0.00), while LH, total testosterone, prolactin, and Inhibin B did not show any statistical significant difference (*P*= 0.065, 0.083, 0.257, 0.268, respectively) [data not shown]. No statistically significant difference was found between SCO and HS patients regarding FSH, LH, total testosterone, prolactin or Inhibin B (*P*=0.05, 0.804, 0.245, 0.423, respectively) [data not shown]. In addition, all hormones did not show any statistical significant difference in their values when HS and SGA patients were compared (data not shown). Consequently, hormonal levels cannot predict the histology of testicular biopsies.

4.5. Cytogenetic analysis by GTG banding

Among the 85 infertile men studied, 8 patients showed abnormal chromosomal karyotypes corresponding to a prevalence of 9.4%, the results of cytogenetic analysis is shown in **Table 4.7**. The occurrence of chromosomal abnormalities was only confined to the azoospermic patient group. Patients with abnormal karyotypes represented 13.6% of the azoospermic patients. Four had a 47, XXY karyotype (Cases# 9, 27, 59, and 74), correspondent to Klinefelter's syndrome, and represented 4.7% of the patient study population, 50% of the chromosomal abnormalities patient group, and 6.8% of the azoospermic patient group (**Fig. 4.1.**). One patient (case# 46) had 47,XY, +mar karyotype, and represented 1.18% of the patient study population, 12.5% of the chromosomal abnormalities patient group, and 1.7% of the azoospermic patient group (**Fig. 4.2**). One patient (case# 64) had

46,XY, del 17(q25) karyotype, and represented 1.18% of the patient study population, 12.5% of the chromosomal abnormalities patient group, and 1.7% of the azoospermic patient group (**Fig. 4.3.**). One patient (case# 69) had a chromosomal instability with a heterogeneous scale of mosaic aneuploides, (**Fig. 4.4-4.6.**). One patient (case# 86) had Robertsonian fusion between chromosomes 15 and 21 [45,XY, -15,-21,+Robertsonian Fusion (15;21) karyotype] (**Fig. 4.7.**).

Table 4.7. Abnormal cytogenetic results among patient population

Case #	Karyotype	Sperm count	Testicular Pathology
9	47,XXY	Azoospermia	SCO
27	47,XXY	Azoospermia	SCO
46	47,XY,+mar	Azoospermia	ND*
59	47,XXY	Azoospermia	SCO
64	46,XY,del17(q25)	Azoospermia	SGA
69	Chromosomal instability (multiple karyotypes)	Azoospermia	ND
74	47,XXY	Azoospermia	SGA
86	45,XY, -15,-21,+Robertsonian Fusion (15;21)	Azoospermia	ND

*ND=Not Done

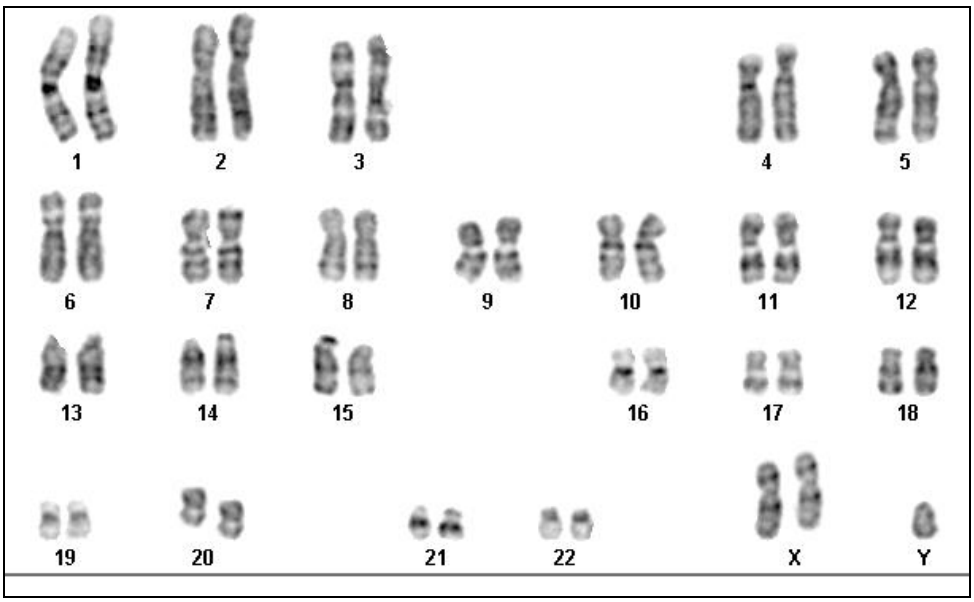

Figure 4.1. Karyotype of one Klinefelter's syndrome patient showing 47,XXY (case#27)

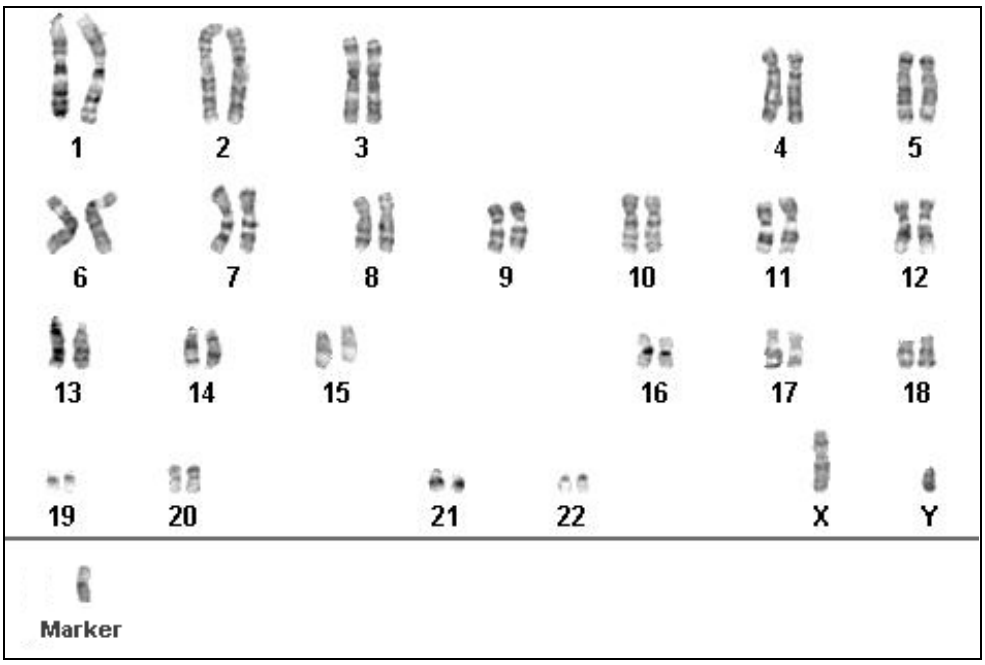

Figure 4.2. 47,XY,+mar karyotype showing extra marker chromosome (case# 46)

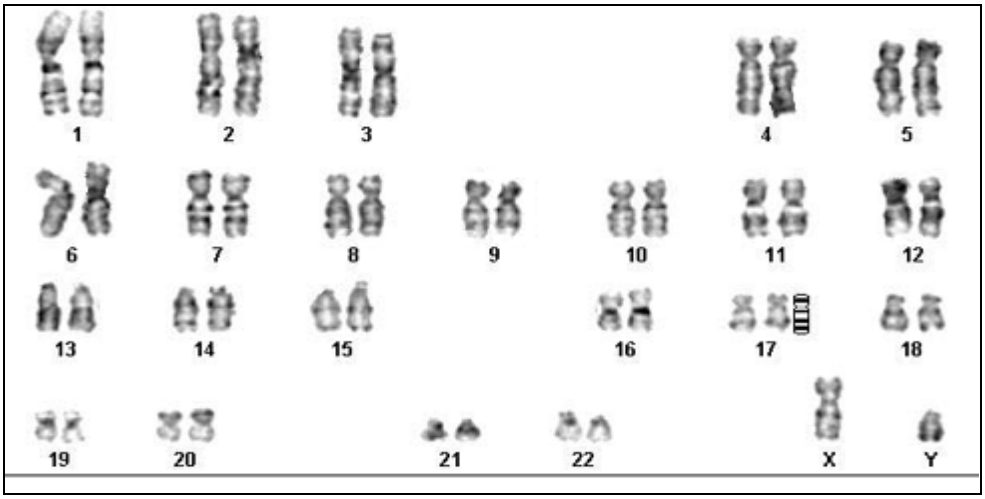

Figure 4.3. 46,XY, del(17)(q25) karyotype (case# 64)

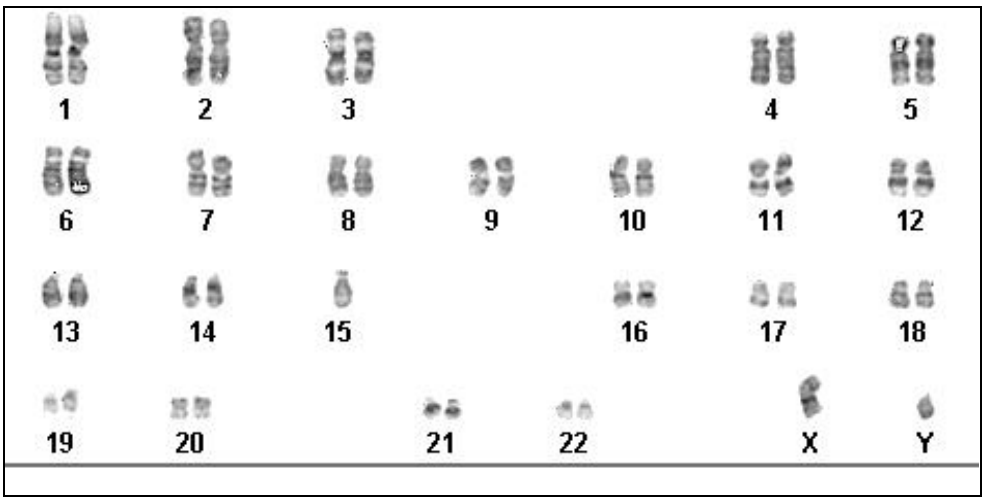

Figure 4.4. 45,XY,-15 karyotype (monosomy 15) [case# 69]

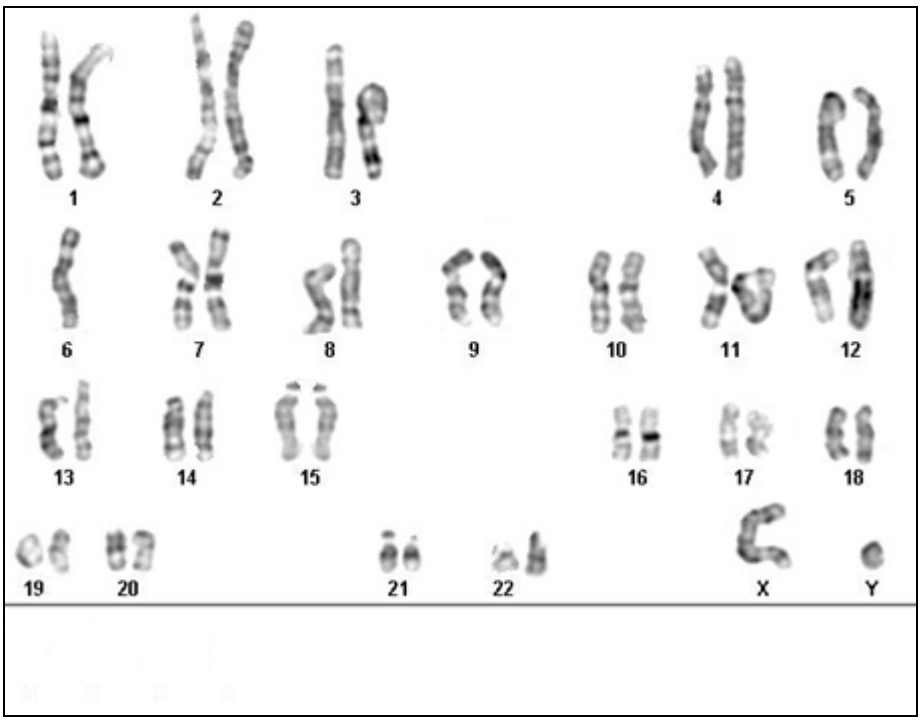

Figure 4.5. 45,Xr(Y),-6 karyotype (case#69)

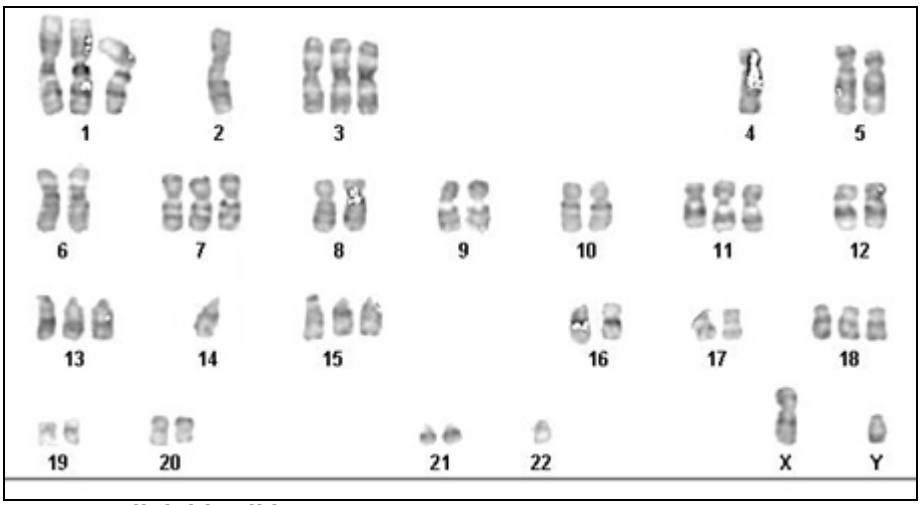

Figure 4.6. Hyperdiploid cell karyotype: 49,XY,+1,-2,+3,-4,+7,+11,+13, -14, +15, +18, -22 (case# 69)

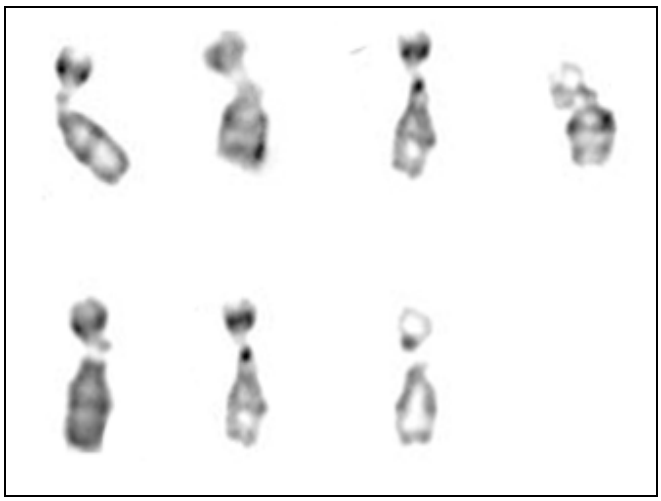

Figure 4.7. Robertsonian fusion (15;21) obtained from 7 different metaphases (case# 86)

4.5.1 Klinefelter's syndrome patients

Patients with Klinefelter's syndrome (KFS) diagnosed in our study (n=4) all showed elevated FSH and LH concentrations and ranged from 27.0 to 43.0 (normal range 2-10 mIU/ml) and 16.8 to 31.6 (normal range 1.0-13.8 mIU/ml), respectively. Serum testosterone values ranged from 1.6 to 2.4 (normal range 2-7 ng/ml) were all either below the normal range or in the low normal range. All of our patients were azoospermics, and all had small testes.

Our Klinefelter syndrome patient series showed significantly higher FSH and LH values than the other azoospermic patients (*P*=0.005, and 0.00 respectively). Their heights also were significantly higher than the other azoospermic patients (*P*=0.004) as well as the entire patient population (*P*=0.001). While serum testosterone levels and semen volumes were significantly lower than the other azoospermic patients (*P*= 0.028 and 0.005, respectively) or the entire population (*P*=0.001 and 0.005, respectively). **Table 4.8** shows all of the aforementioned *P* values. It was also observed that none of our patients had a history of neither varicocele nor cryptorchidism. All patients have SCO testicular biopsy, except one who showed SGA biopsy result.

Table 4.8. Comparisons between KFS patients and different spermatogenic potential subgroups

Group	FSH	LH	Test[c]	PRL[d]	Height	Weight	Volume[e]
Entire population [a]	0.001[b]	0.0[b]	0.014[b]	0.752	0.001[b]	0.172	0.005[b]
Azoospermics	0.005[b]	0.0[b]	0.028[b]	0.916	0.004[b]	0.179	0.005[b]
Cryptozoospermics	0.215	0.184	0.017[b]	0.29	0.161	0.284	0.188
Severe Oligozoospermics	0.00[b]	0.00[b]	0.003[b]	0.602	0.004[b]	0.151	0.005[b]
Oligozoospermics	0.00[b]	0.002[b]	0.014[b]	0.334	0.015[b]	0.601	0.080[b]

[a] Excluding Klinefelter's patients;
[b] Tagged *P* values indicate statistical significance (*P*<0.05)
[c] Total Testosterone
[d] Prolactin
[e] Semen volume

4.6. *AZF* classical microdeletions

No Y chromosome classical microdeletions in *AZFa*, *AZFb* or *AZFc* could be detected among the 85 infertile men included in this study. An example of the STSs adopted to detect classical microdeletions and their product sizes are shown in **Fig. 4.8**.

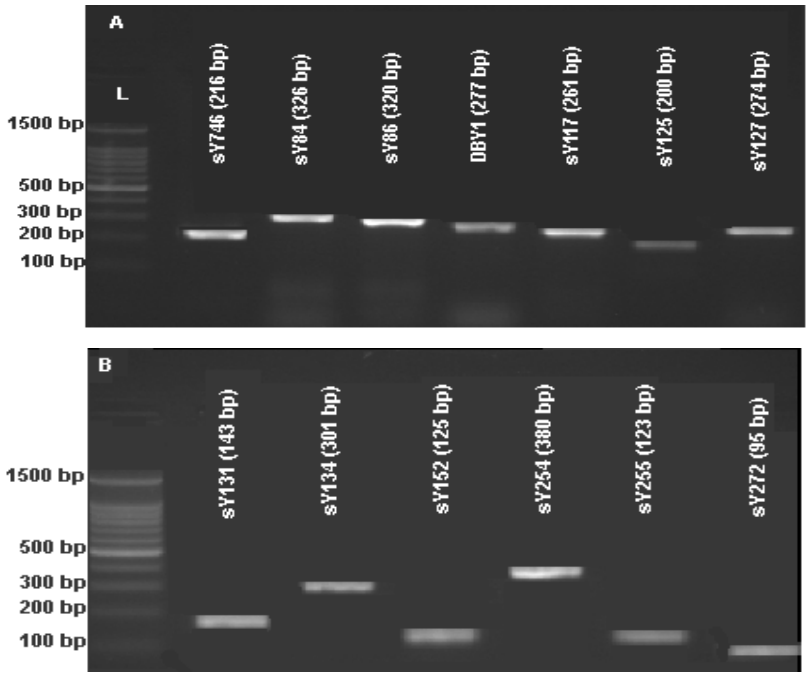

Figure 4.8. A and B are examples of ethidium bromide stained agarose gels for detecting classical *AZF* deletions and the STSs and their product sizes adopted in the study for case# 83. L: 100 bp DNA Ladder.

4.7. Distribution of partial *AZFc* deletions in patients and proven fertile controls

In order to detect partial *AZFc* deletions our goal was to detect the unique fragments flanking the *DAZ*1/*DAZ*2 doublet at the u3 segment (proximal) and the P2/P1 palindrome junction (distal), corresponding, respectively, to sY1191 and sY1291 used in previous studies (Repping *et al.*, 2003; Fernandes *et al*., 2003; Machev *et al*., 2004). We relied on sY1191 and sY1291 STSs to discriminate between the different *AZFc* partial deletion patterns, by applying the results shown in **Table 4.9**.

Table 4.9. +/- STS PCR *AZFc* partial deletion classification scheme

AZFc partial deletion pattern	sY1291	sY1191
No deletion	+	+
gr/gr	-	+
b2/b3	+	-
b1/b3	-	-

(+)= No deletion; (-)= deletion

In total, seven (8.2%) out of the 85 infertile men investigated had partial deletions within the *AZFc* region. In particular, we found two different patterns of partial *AZFc* deletions; the gr/gr (6/85, 7%) and the b1/b3 (1/85, 1.2%) deletions, whereas b2/b3 was absent in our patient group. One man with gr/gr deletion was oligozoospermic (case# 57, sperm count 6.4 M/ml), one was severely oligozoospermic (case# 79, sperm count 0.7 M/ml), but the others were azoospermics, while the patient with b1/b3 deletion was severely oligozoospermic (case# 71, sperm count 2.6 M/ml). Testicular biopsy reports were available only for three of the seven patients with partial deletions of the *AZF*c region. One patient (case# 17) had Sertoli cell-only syndrome (SCOS), one patient (case# 47) had spermatogenic arrest and one patient (case# 64) had severe hypospermatogenesis. One subject (1/30, 3.3%) in the control fertile group proved to have gr/gr deletion, which was the only pattern of partial deletions of the *AZF*c region observed in the control group. The summary of all deletions and their distribution in the different groups of

spermatogenic failure are given in **Table 4.10**. The clinical data of the deleted subjects in the infertile group are shown in **Table 4.11**. The sY1291 and sY1191 STS PCR results for some patients with partial *AZFc* deletions are shown in **Figures 4.9 and 4.10**.

Table 4.10. Frequencies of partial *AZFc* deletions in the study population and the control groups

Group		Deletion Pattern			
	n	gr/gr; n(%)	b2/b3; n(%)	b1/b3; n(%)	Total; n(%)
Infertile	85	6 (7.0%)	None	1 (1.2%)	7 (8.2%)
Control	30	1 (3.3%)	None	None	1 (3.3%)

Table 4.11. Clinical data of subjects with partial *AZFc* deletions

Case #	Deletion Pattern	Sperm Count¶	Testicular Biopsy§	FSH mIU/ml	LH mIU/ml	Test$ ng/ml	PRL£ ng/ml	Inhibin B pg/ml
17	gr/gr	Azo	SCO	19.3	8.2	5.7	8.6	36
47	gr/gr	Azo	SGA	7.5	8.0	5.0	17.0	NA¥
49	gr/gr	Azo	ND	12.0	5.3	2.8	15.0	NA
57	gr/gr	Oligo	ND	4.5	13.2	4.7	16.1	NA
64	gr/gr	Azo	HS	2.4	NA	1.4	NA	NA
71	b1/b3	S Oligo	ND	NA	NA	NA	NA	NA
79	gr/gr	S Oligo	ND	6.1	9.2	5.2	20.4	NA

¶ Azo= Azoospermia, Oligo= Oligozoospermia, S Oligo= Severe oligozoospermia
§ SCO= Sertoli cell-only, SGA= Spermatogenic arrest, HS= Hypospermatogenesis, ND= Not done
¥ NA= Not Available
$ Testosterone
£ Prolactin

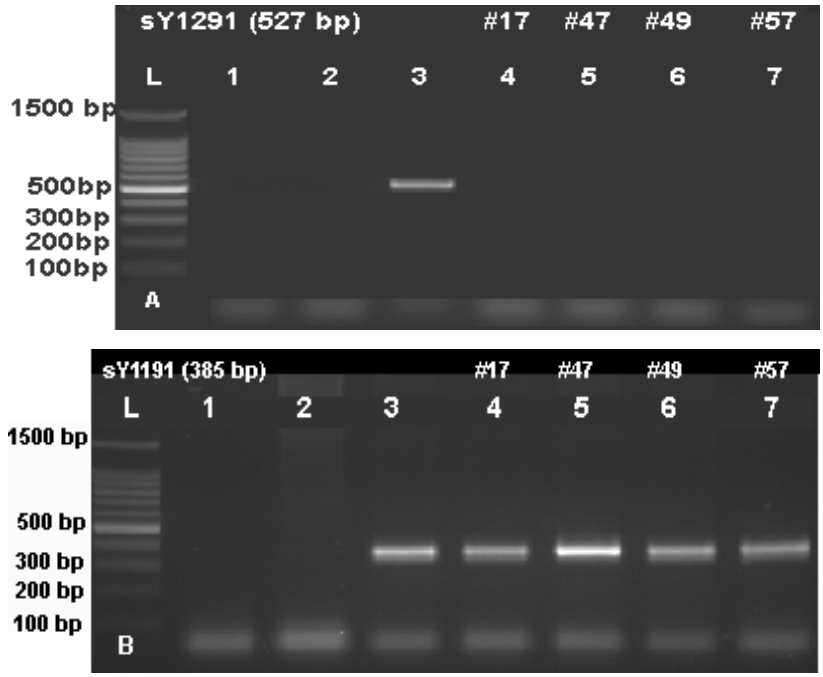

Figure 4.9. Ethidium bromide stained agarose gels for some of our gr/gr deleted cases. A: sY1291 STS PCR reactions demonstrating absence of sY1291 (527 bp) in gr/gr deletion cases #17, 47, 49, and 57 (lanes 4-7, respectively). B: sY1191 STS PCR reactions showing presence of sY1191 (385 bp) in the same aforementioned gr/gr deleted cases (lanes 4 to 7 respectively). L= 100 bp DNA ladder, Lane 1: negative control (water), Lane 2: negative control (normal female genomic DNA), Lane 3: positive control (normal proven fertile male genomic DNA).

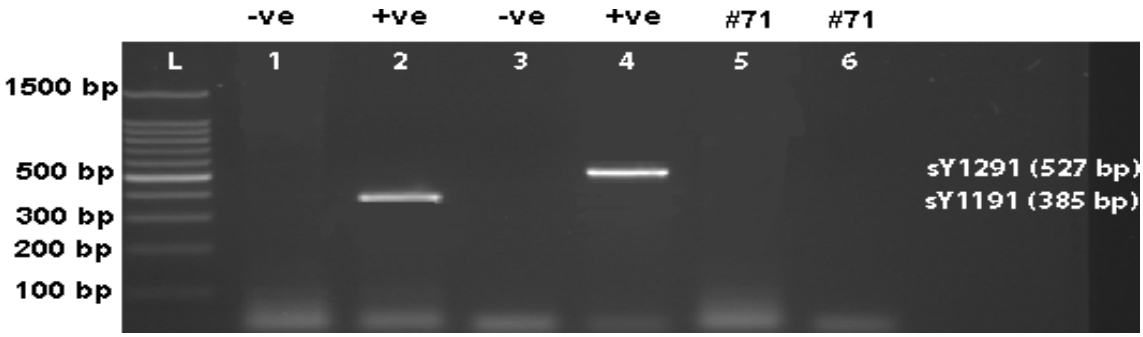

Figure 4.10. Ethidium bromide stained agarose gel electrophoresis for case# 71 that harbors b1/b3 partial *AZFc* deletion. L: 100 bp DNA Ladder, Lane1: sY1191 negative control (normal female genomic DNA), Lane2: sY1191 positive control (normal proven fertile male genomic DNA), Lane3: sY1291 negative control (normal female DNA), Lane 4: sY1291 positive control (normal proven fertile male DNA), Lane 5: sY1191 for case# 71, Lane 6: sY1291 for case# 71. Both sY1191 and sY1291 are absent in case# 71 and this characterizes b1/b3 partial *AZFc* deletion according to the +/- partial *AZFc* classification scheme.

The gr/gr deletion was found in both the infertile and the proven fertility control populations. The deletion frequency of gr/gr in the infertile group was 7.0%, while it was 3.3% in the control proven fertile group. No statistically significant difference in deletion frequency was found between the two populations (χ^2= 0.54, *P*= 0.764,).

The deletion frequency of b1/b3 in the infertile group was 1.2%, while it was 0.0% in the proven fertility control group. The difference in deletion frequency between the two populations was not statistically significant (χ^2= 0.356, χ^2 critical=3.84, α = .05).

In addition, no statistically significant difference in partial *AZFc* deletion frequency was found between the infertile and the proven fertility control populations (χ^2= 0.8233, *P*=0.663).

No statistically significant difference was found between the partially deleted *AZFc* patients and the nondeleted infertile patients in levels of FSH (*P*=0.061), LH (*P*=0.54), total testosterone (*P*=0.585), prolactin (*P*=0.239), or Inhibin B (*P*=0.5), as well as in height (*P*=0.753), weight (*P*=0.618), and seminal fluid volume (*P*=0.087). Consequently, hormonal data are not useful in predicting cases with lone absence of sY1291.

No family history of infertility was noted for all *AZFc* partially deleted patients, except one (case# 79, sperm count 0.7 M/ml) in which his brother also suffers from severe oligozoospermia (sperm count 0.4 M/ml) that was tested for partial *AZFc* deletions, and his Y chromosome was also found to harbor gr/gr deletion, see **Figure 4.11** lane 11. Moreover, his uncle (from his mother side) suffers from infertility (no data available, but he is infertile according to the WHO definition), suggesting that his infertility is inherited and it is most probably related to the autosomes or the X chromosome, but not to the Y chromosome.

4.7.1. Source of *AZFc* partial deletions

In all instances in which DNA samples from the father (case# 17), or the brothers (cases# 49, 64, 79) of an infertile gr/gr deletion subject were available for testing, we found that the father's and brothers Y chromosomes also had gr/gr deletion, provided that all of the brothers are married and all had at least naturally conceived one child, except case# 79 (see above), **Figure 4.11**. No relatives were available for testing the source of the b1/b3 partial deletion found in Case# 71.

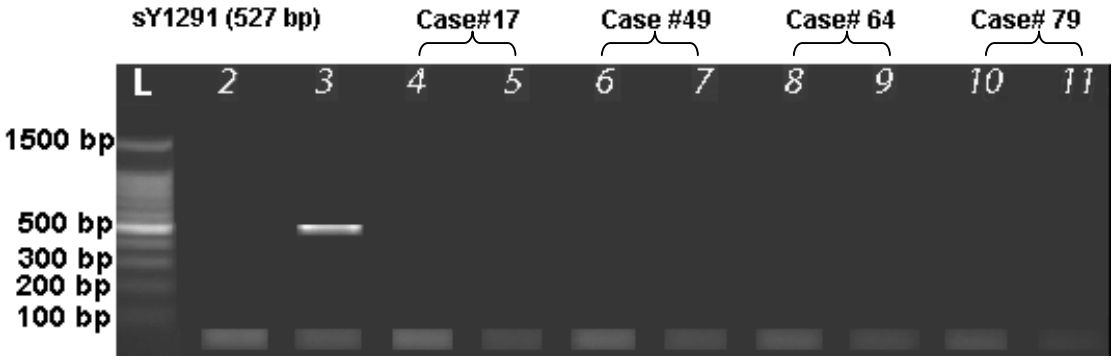

Figure 4.11. Ethidium bromide stained agarose gel electrophoresis for determining the source of the gr/gr deletion pattern in our patients. L: 100 bp DNA Ladder, Lane 2: negative control (normal female genomic DNA), Lane 3= positive control (proven fertile male genomic DNA), Lane 4: case #17, Lane 5: father of case# 17, Lane 6: case# 49, Lane 7: brother of case# 49, Lane 8: case# 64, Lane 9: brother of case# 64, Lane 10: case# 79, Lane 11: brother of case# 79.

Chapter Five

DISCUSSION

5.1. Cytogenetic analysis by GTG Karyotyping

In the present study, both numerical and structural chromosomal aberrations were found in our patient study population. The occurrence of the chromosomal abnormalities was only confined to the azoospermic patient group; this could be the effect of the small size and slightly unbalanced nature of our patient study population (azoospermics 59, severely oligozoospermics 17, and oligozoospermics 6). The prevalence of chromosomal anomalies among the studied infertile men was found to be 9.4%. This figure lies within the previously published range (3.6-22.6%), as shown in **Table 5.1**. Association between human male infertility and chromosomal anomalies has been known for a long time (Chandley A, 1979; Faed *et al*., 1979). Chromosomal abnormalities are more frequently observed in the population of azoo/oligozoospermic males than in the general population (Tuerlings *et al*., 1998). Thus, it would not be unusual to find chromosomal abnormalities in men attending infertility clinics.

Table 5.1. Literature reporting chromosomal abnormalities in infertile men

Source	Study subjects (n)	Chromosomal abnormalities (%)
Kondoh *et al*., 1992	130	13.8
Pandiyan and M-Jequier, 1996	1210	3.6
Gündüz *et al*., 1998	102	15.7
Kleiman *et al*., 1999	72	16.6
Penna Videaú *et al*.,2001	84	22.6
Nagavenker *et al*., 2005	88	10.2
Pina-Neto *et al.,* 2006	165	9.6
Hellani *et al*., 2006	257	3.9
Mohammed *et al*., 2007	289	8.0
This study, 2007	85	9.4

The distribution of chromosomal abnormalities detected in the present study showed that Klinefelter's syndrome (47,XXY) was the most prevalent abnormality, representing 50% (4/8) of our positive cases. This result was in agreement with several previously published studies (Guichaoua *et al*., 1993; Pandiyan and M-Jequier, 1996; Nakamura *et al*., 2001; Penna Videaú *et al*., 2001; Rao *et al*., 2004; Lissitsines *et al*., 2006; Pina-Neto *et al*., 2006; Mohammed *et al*., 2007). Our result was not unexpected since Klinefelter's syndrome was described as the most frequent genetic cause of male infertility (Lanfranco *et al*., 2004; Simoni *et al*., 2004).

All KFS patients have SCO testicular biopsy, except one who showed SGA biopsy result; this is not unexpected result since there are several reports in which Klinefelter's syndrome patients have fathered biologically although only by assisted reproduction techniques (Tournaye *et al*., 1996; Bergere *et al*., 2002). This proves that not all Klinefelter's syndrome patients deem to be a homogenous group. Therefore, testicular biopsies and/or TESE should be warranted for all Klinefelter's syndrome patients. The specific combination of azoospermia, very high FSH and LH, small testes, tall stature, low or low normal testosterone and reduced semen volume may predict the XXY phenotype.

The exact mechanism by which chromosomal anomalies induce infertility is not clear. It is likely that the presence of abnormally distributed chromatin may interfere with meiotic division, therefore, reduces sperm production (Yamamoto *et al*., 2002). Testicular histology in such patients may reveal areas of atrophy and hyalinization of the seminiferous tubules as well as some areas with tubules of normal appearance that contain a reduced number of mature spermatozoa (Yamamoto *et al*., 2002; Wikstrom *et al*., 2004). Spermatozoa bearing abnormal chromosomes may cause abnormal embryonic development that can in turn cause early pregnancy loss (Evans *et al*., 1978). Moreover, these chromosomal aberrations may have serious implications for infertile males who seek the help of intra-cytoplasmic sperm injection (ICSI) due to the possibility of transmission of these abnormalities to the offspring (Reubinoff *et al*., 1998; Ron-el *et al*., 1999; Bonduelle *et al*., 2005).

To conclude, chromosomal abnormalities found with relatively high prevalence in our infertile males are the major cause of their male infertility, and justify the requirement of cytogenetic analysis for every infertile male, particularly azoospermics, seeking children.

5.2. Y-chromosome classical microdeletions

The present study shows absence of *AZF* microdeletions in the long arm of the Y chromosome in the studied infertile population. This is in agreement with some previously published studies. Tzschach *et al*. (2001) reported the absence of such structural abnormality in 97 infertile men, Gruber *et al*. (2003) in 383 infertile men, and Bush *et al*. (2004) in 96 infertile men. Moreover, very low prevalence of Y chromosome microdeletions was reported in other studies. Bor *et al*. (2002) reported a prevalence of 0.75% of Y chromosome microdeletion in 400 infertile men, Medica *et al*. (2005) found Y chromosome microdeletions in 0.95% of 105 infertile males and Van der Ven *et al*. (1997) found Y chromosome microdeletion in 0.98% of 204 infertile.

Nap *et al*. (1999) reported deletions in 2.3% of 1627 infertile men who were seen in 14 infertility centers in the Netherlands and Belgium, and Ferlin *et al*. (2007) in a prospective study that extended for 10 years (from January 1996 to December 2005) reported a total incidence of 3.2% in 3073 infertile men. However, the same authors in 1998 reported the highest proportion of microdeletions published so far, they reported an incidence of 55% among a highly selected group of 18 patients with histologically confirmed Sertoli cell-only syndrome (SCOS). More intriguing, Kihaile *et al*. (2005) studied the occurrence of Y chromosomal microdeletions in two different populations, Japanese and Africans. They found Y chromosome microdeletion prevalence of 6.2% in Japanese, but absence of Y chromosome microdeletions in Africans. This may highlight the association of Y chromosomal microdeletions with certain Y chromosome haplogroups. Some Y haplogroups are more susceptible to deletions than others, while other Y chromosome haplogroups may confer protection against microdeletions. For example, Y chromosome haplogroup J seems to protect against *AZFa* deletions because they lack the LIPA4 element in HERV15q2 which is supposed to facilitate the homologous intrachromosomal recombination leading to *AZFa* deletion as described by

Ferlin *et al*. (2007). Moreover, same investigators also observed a reduction of the J haplogroup frequency in their *AZFc* microdeleted samples. Noteworthy, Nebel *et al*., (2001) found a high proportion of Palestinians (55.2%), residing in Israel and the Palestinian Authority area to belong to this particular Y haplogroup. Furthermore, they also observed that Palestinians differ in there Y chromosome pool from Europeans and other Middle East populations included in their study.

It was postulated by Vogt (2005a) that the number of amplicons and palindromes in the *AZFb* and *AZFc* regions vary between different Y chromosome haplogroups. If this holds true, then we can safely assume that the number of amplicons and palindromes in the present studied Palestinian infertile males Y chromosome haplogroup(s) most likely differ or deviate from the established reference *AZFb* and *AZFc* structures that belong only to one donor (RP-11 donor), and thus to one Y chromosome haplogroup, i.e., R^{*} Y haplogroup, which is not popular in our Middle East region. Noteworthy, this single donor fertility status is unknown.

During our extensive search in the literature concerning Y chromosome microdeletions, we found only five Arab studies. The first one was conducted in Egypt (El Awady *et al*., 2004), which is our direct neighbor country; they reported a frequency as high as 12% (4/33), two with *AZFc* deletion, one with *AZFa* deletion, and one with *AZFa-b-c* deletion. The two patients with *AZFc* deletion described in the study were found to be deleted only for a single STS (sY272) and not for the other *AZFc* STSs they used, and the authors suggested that this STS might be considered as a hot spot site for Y chromosome microdeletions in Egyptian population. If we take into account Pryor *et al*. (1997) findings regarding this particular STS in which they found deletion of this single STS in two normal males who have conceived children before as well as in one infertile male whose father also shows an absence of the same STS. This proves that this STS (sY272) is a polymorphic site and its absence can be regarded as a polymorphic loss and not as deletion. This will substantially reduce the frequency of microdeletions in the Egyptian study to 6%. In addition, their small population size should also be taken into consideration, as each patient included in their study population represents 3%. The second study was conducted among Saudis in Saudi Arabia (Hellani

et al., 2006) where the authors reported incidence was 3.2% (8/247). The authors concluded that Y chromosome microdeletions are low in Saudi population. The third study was Tunisian (Hadj-Kacem *et al*., 2006), which had shown a prevalence of 16% (26/163) in a group of patients who had sperm counts ranging from normospermia (65/163), oligozoospermia (53/163) to azoospermia (45/163). If we exclude the normospermics then the frequency will be raised to 26.5%, i.e., one out of 4 patients; moreover they stated that they detected a deletion, uncharacterized by the authors, in one normospermic infertile patient. It is noteworthy that they had used only 8 STSs to detect such deletions, and we believe that the results of this Tunisian paper are conflicting. The fourth study was conducted among Moroccan infertile men (Imken *et al*., 2007); the authors reported prevalence was 3.2% (4/127). The fifth study was a novel study that was conducted among Kuwaitis in Kuwait (Mohammed *et al*., 2007) where the authors reported a low *AZF* microdeletion prevalence of 2.4% (7/266). The low prevalence of microdeletions in both Kuwaitis (2.4%) and Saudis (3.2%) may reflect the common ethnic origin, as both are next-door neighbors.

To summarize, we suggest that the low prevalence of *AZF* classical deletions is influenced by ethnic factors, genetic background and Y chromosome haplogroups. Therefore, we strongly recommend conducting studies to analyze and then determine the distribution of Y-chromosome haplogroups in Gaza Strip population. Moreover, other genetic, epigenetic, nutritional and environmental factors may be responsible for the oligozoospermia and azoospermia cases observed in the Gazian Palestinian population.

5.3. *AZFc* partial deletions

In the present study, we analyzed the occurrence of partial *AZFc* deletions in Gazian Palestinian infertile men and proven fertile controls. The commonest partial *AZFc* deletion pattern observed was the gr/gr pattern which was detected in six (6/85, 7%) patients and in only one (1/30, 3.3%) proven fertile control; there was no statistically significant difference between the two groups. This figure (7%) is slightly higher than the previous reported figures of about 5% in infertile men (Repping *et al*., 2003; de Llanos *et al*.,

2005; Fernando *et al*., 2006). Nevertheless, small population sizes usually show higher frequencies.

In three gr/gr deleted patients, the origin of the deletion was confirmed to be inherited and not due to a *de novo* rearrangement, as their fertile relatives showed the same deletion pattern. The fourth available relative was the brother of case# 79, who showed the same deletion pattern as his brother, i.e. gr/gr deletion pattern, giving that he is severely oligozoospermic. This particular case may suggest that the Y chromosome is not responsible, since their uncle from mother side, no data available, is also infertile but according to the WHO definition. The successful transmission of gr/gr deletion pattern from generation to generation contraindicates a direct association of gr/gr deletion and spermatogenic failure. Our data show that gr/gr deletion represents a polymorphic event as it is found not only in patients suffering from spermatogenic failure but also in men with proven fertility.

As mentioned earlier, 55.2% of Palestinians belong to the J haplogroup as reported by Nebel *et al*. (2001). Machev *et al*. (2004) had described polymorphic loss of sY1291 locus in J haplogroup. gr/gr deletions are characterized by the deletion of either *DAZ* gene doublets, whereas polymorphic loss of sY1291 does not alter the *DAZ* copy number. Therefore, the absence of only sY1291 in not sufficient in confirming gr/gr deletion, for confirmation, we recommend the extension of this study using FISH, Southern blot, and quantitative *DAZ* copy assay.

Interestingly, both Lin *et al.* (2007) and Zhang *et al*. (2007) had described individuals who lack sY1291 amplified fragments but with 4 *DAZ* genes. These individuals where found to carry a Y chromosome variant with a gr/gr deletion followed by an additional compensatory duplication of b2/b4 *AZFc* region that restored the *DAZ* copy number. This del/dup *AZFc* model can be proved by the presence of only 2 kinds of *DAZ* instead of 4 kinds (as each *DAZ* differ in the RNA-binding motif copy number), or measuring *DAZ* copy number by quantitative *DAZ* copy assay.

In the present study, we found no case with complete *AZFc* deletion. Zhang *et al*. (2007) assumed that complete *AZFc* deletion in addition of being caused by a *de novo* complete deletion, it could be caused by a two step process, partial *AZFc* deletion followed by deletion of the entire *AZFc* region.

They also postulated that a high frequency of complete *AZFc* deletions in Y-chromosomes harboring gr/gr deletion is expected. If this holds true, and if our cases are true gr/gr deletion cases, consequently, we will expect a high complete *AZFc* deletion frequency in our population, however, this was not the case!

Alternatively, we believe that our sY1291 deleted cases in the infertile as well as in the proven fertile groups are either due to the polymorphic loss of sY1291 locus representing untrue gr/gr deletions, or due to the initial gr/gr deletion followed by a compensatory partial duplication of the b2/b4 *AZFc* region, i.e., del/dup *AZFc* model, or both. Furthermore, Y haplogrouping should be defined on these cases to explore if a specific Y chromosome haplogroup have a fixed gr/gr deletion that have been descended from a deleted founder(s) or not.

The b1/b3 deletion pattern, a 1.6 Mb deletion size, was only observed in the infertile group, but because we could not detect more than one case (sperm count 2.6 M/ml) it was not sufficient to draw conclusions on its effect on spermatogenesis. In addition, due to the shortage of DNA for this particular case we couldn't do further characterization, but since it is characterized by the absence of both sY1191 and sY1291, which are apart, we would safely regard it as true (pathogenic) deletion, in appose of our sY1291 deleted cases.

As a conclusion, our results and observations are congruent with Machev *et al*. (2004), Hucklenbroich *et al*. (2005), Ravel *et al*. (2006), Carvalho *et al*. (2006) and Lin *et al*. (2007) in that the gr/gr microdeletion, at least in our population, are neutral variants with no phenotypic effect. There is a pressing need for Y-chromosome haplogroup and gr/gr analysis in large groups of normospermic and azoo/oligozoospermic men of different ethnic origins to characterize and explore the role played by this type of microdeletion in male infertility. Moreover, performing Southern blot analysis on the *DAZ* genes or quantitative *DAZ* copy assay (real-time PCR) and *DAZ*-SNV, at least in our gr/gr deletion pattern positive cases, to determine the RNA–binding motif (RBM) copy number in the various *DAZ* genes and *DAZ* copy number, in order to prove or exclude the possibility of del/dup *AZFc* model or ascertaining *DAZ* gene doublet deletion in these cases, respectively. In addition, sequence tagged sites +/- (+/- STS) PCR *AZFc* classification scheme may need further

refinement to exclude the possibility of a polymorphic loss of a single marker in particular sY1291.

Chapter 6

CONCLUSIONS and RECOMMENDATIONS

6.1 Conclusions

The main aim of the study was to determine the prevalence of numerical and structural chromosomal abnormalities among idiopathic azoospermic and oligozoospermic infertile males in Gaza Strip. The conclusions that have been drawn from the present study include:

- The overall prevalence of genetic abnormalities at the cytogenetic level in Gazian Palestinian infertile population is 9.4%, urging the inclusion of cytogenetic testing in each diagnostic workup of any infertile man.
- Klinefelter's syndrome is the most prevalent genetic cause of male infertility in our population and that this syndrome is not always associated with SCO.
- The specific combination of azoospermia, very high FSH and LH, small testes, tall stature, low or low-normal testosterone and reduced seminal fluid volume may predict the XXY phenotype.
- FSH levels are in direct relationship with degree of spermatogenic failure.
- The frequency of classical *AZF* deletions is very low in our studied patients; consequently, inclusion of the *AZF* classical microdeletion assay in male infertility workup in our population is obviously questionable.
- Hormonal data are not useful in predicting cases with lone absence of sY1291.
- In our population, gr/gr partial *AZFc* deletions are not associated with spermatogenic failure.
- Hormonal parameters cannot predict the testicular histology.

6.2 Recommendations

According to the results and observations of the current study we recommend the followings:

1- Cytogenetic testing is mandatory and should be included in any male infertility diagnostic workup.
2- Testicular biopsies and/or TESE should be warranted for all Klinefelter's syndrome patients, as they are representing a non-homogenous group.
3- There is a pressing and urgent need for defining Y-chromosome haplogroups in our Palestinian population, in both infertile and fertile groups.
4- Using gene-specific markers instead of anonymous STSs as an alternative approach of testing Y-chromosome microdeletions should be initiated to detect deletions or mutations that may escape detection, or lying upstream or downstream the adopted STSs.
5- We recommend the extension of this study using FISH, Southern blot, and quantitative *DAZ* copy assay for the gr/gr deletion pattern cases.
6- Employing Southern blot analysis, *DAZ*-SNV analysis, and quantitative *DAZ* assay on our gr/gr deleted pattern cases to ascertain whether they are due to *DAZ* gene deletion, or either due to polymorphic loss of sY1291 and/or del/dup *AZFc* model.
7- Defining Y haplogroup(s) of our gr/gr deleted pattern cases to explore if they are associated with certain Y haplogroup(s) or not.
8- +/- STS *AZFc* classification scheme should be revised and refined to exclude the possibility of polymorphic loss of a single locus.
9- Search for other genetic, epigenetic, nutritional and environmental factors causing male infertility in our region should be promptly granted.

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